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SOME STORAGE DISEASES OF GRAPEFRUIT [1]

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INTRODUCTION

The storage life of grapefruit (*Citrus grandis* (L.) Osbeck) is greatly limited because of the development of diseases that produce blemishes in the peel. The present publication reports a study of certain of these diseases under various storage conditions.

DESCRIPTION OF DISEASES

PITTING

Pitting, or "pox", the most serious of the storage diseases of grapefruit, has received major consideration in all previous reports. As the term "pitting" implies, the outstanding characteristic of the disease is the development of pits or depressions in the rind of the fruit. In the present paper, pitting is divided into two classes, the definite and the mild.

The term "definite" is used to refer to pits that are 0.2 inch or more in diameter and that are depressed to a fairly uniform depth of about one-thirty-second of an inch (figs. 1 and 2, *A*). The oil glands are usually depressed to the level of the rest of the pitted tissue and sometimes show slightly greater depression. They are rarely elevated above the rest of the depressed area. The pits are seldom normal in color. Occasionally they appear bleached or faded but usually they are darker than the healthy peel, ranging in color from yellow ocher to ochraceous tawny or tawny.[2] They tend to darken with age, yet color can hardly be considered a good index of age.

The term "mild" is used to refer to pitting that is less pronounced than that described above. The pits are usually less than 0.2 inch in diameter and may be relatively shallow although they are often as deep as the definite pits (fig. 2, *B*). They may occur individually or in groups. They sometimes form a zone of small pits around the definite pits (fig. 3) and they may enlarge or coalesce to form definite pits (fig. 4, *A*), but they should not be regarded merely as a stage in the development of the larger pits. The definite pits usually make their appearance without passing through the mild stage and the storage conditions that are favorable to one type of pitting are not always favorable to the other.

OLEOCELLOSIS

Oleocellosis of grapefruit differs from pitting in that the oil glands stand out in relief above the collapsed tissue and that the affected

[1] Received for publication June 14, 1935; issued April, 1936.

[2] RIDGWAY, R. COLOR STANDARDS AND COLOR NOMENCLATURE. 43 pp., illus. Washington, D. C. 1912.

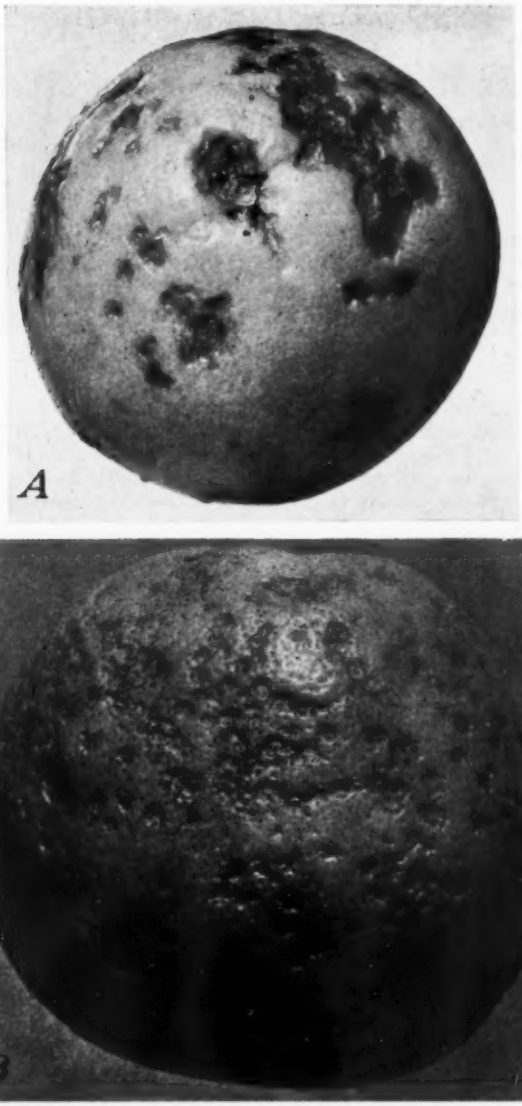


FIGURE 1.—Definite pitting of grapefruit (*A*) at end of 13 weeks' storage at 36° F. beginning May 5, 1933, and (*B*) at end of 2 weeks' storage at 34° F. following shipment from Florida to New York.

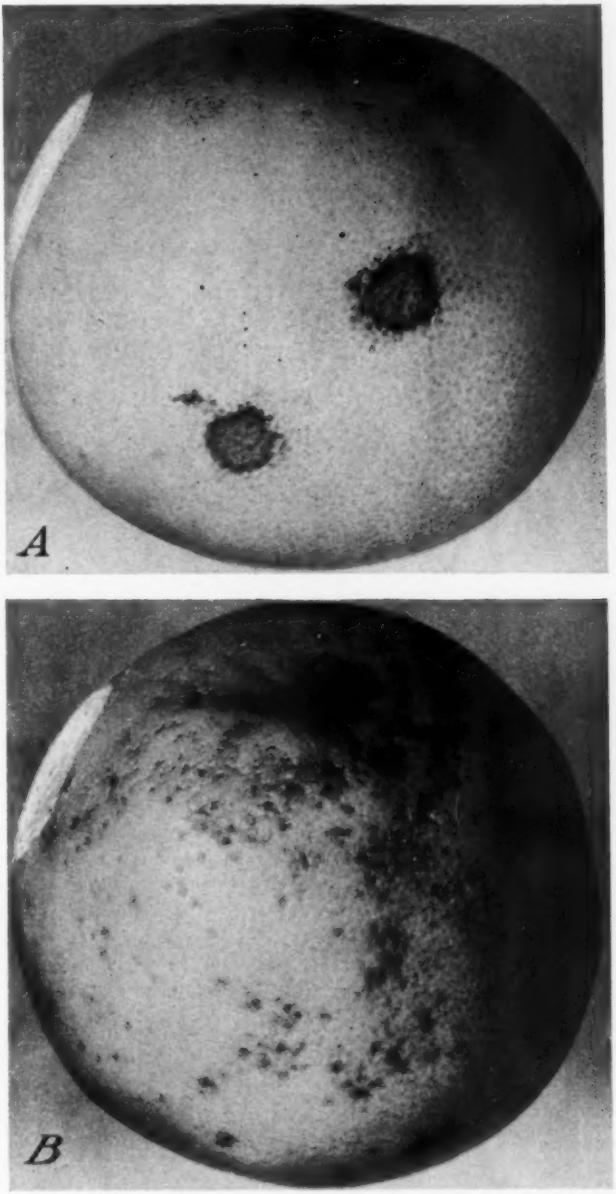


FIGURE 2.—*A*, Definite pitting of grapefruit at end of 6 weeks' storage at 40° F. beginning February 12, 1934; *B*, mild pitting of grapefruit at end of 9 weeks' storage, in glycerin wraps, at 32° F. beginning February 12, 1934.

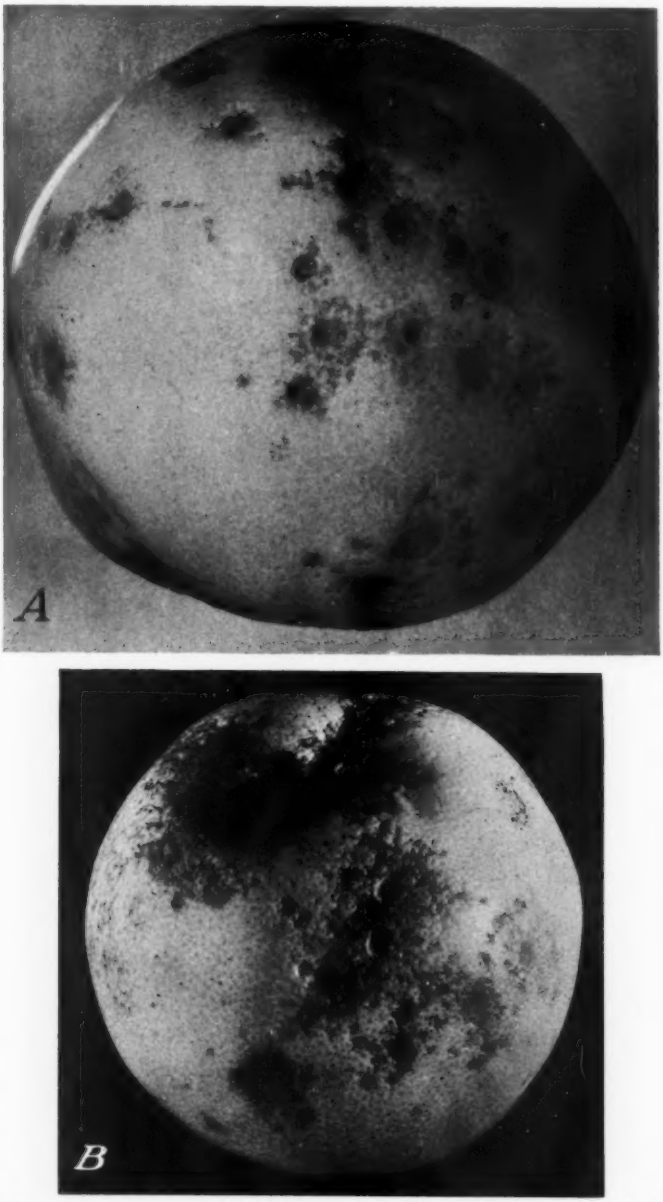


FIGURE 3.—Mild pitting of grapefruit (*A*), forming a zone around definite pitting at end of 6 weeks' storage at 36° F. beginning March 10, 1934, and (*B*), forming groups around definite pitting, at end of 9 weeks' storage at 36° F. beginning February 12, 1934.

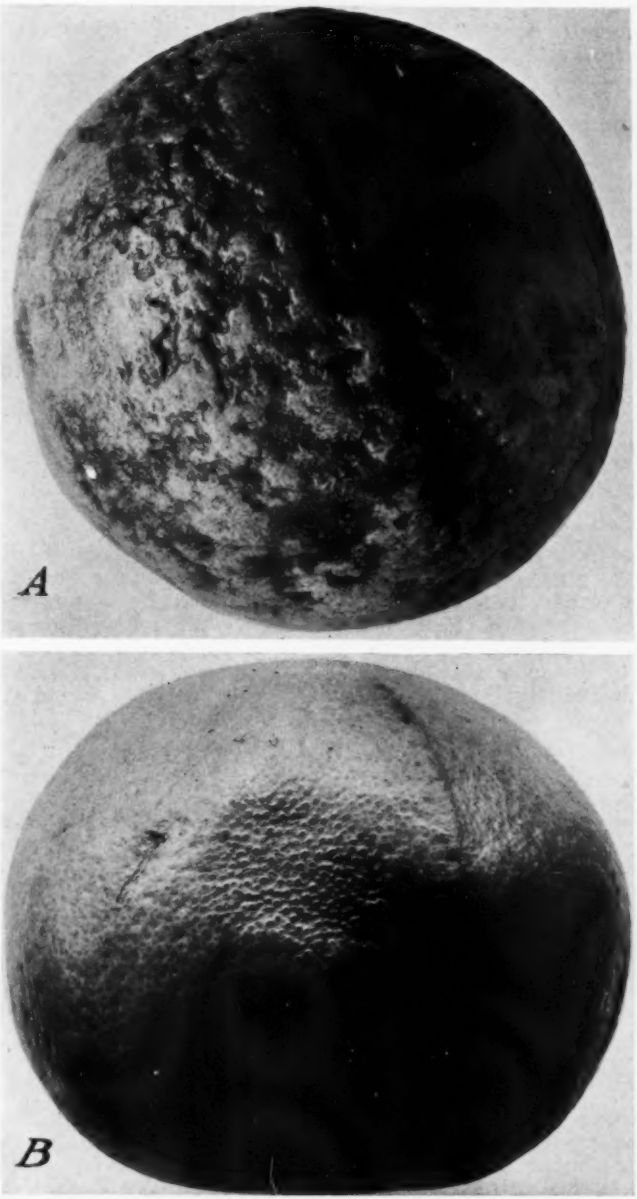


FIGURE 4.—*A*, Mild pitting of grapefruit, coalescing into definite pitting at end of 11 weeks' storage at 32° F. beginning May 7, 1934; *B*, oleocellosis of grapefruit, produced by applying expressed juice from grapefruit peel, followed by 4 weeks' storage at 32° F.

areas are usually but slightly depressed (fig. 4, *B*). Its appearance is similar to that of oleocellosis on oranges and lemons (*4*).[3] As mentioned later, its occurrence is probably initiated by the presence of oil on the surface of the fruit, yet its development may be influenced by storage conditions. Oleocellosis that appears to be due to packing injuries is sometimes referred to as "box scald" or "box rub."

SCALD

In addition to pitting, brown stain has been mentioned as a cold-storage disease of grapefruit by Ramsey (*12*) and scald has been reported by Friend and Bach (*5*). It would seem probable that the two terms refer to the same disease. In the present publication the term "scald" is used in reference to a superficial and fairly uniform browning covering relatively large areas of the peel (pl. 1, *A*). The disease stands out in marked contrast to all forms of pitting, as the affected areas are not depressed. In mild and typical cases the surface of the peel is firm but in severe cases it may become spongy and soft, suggesting an early stage of watery break-down, which is described later. The color of the scald areas ranges all the way from that of the normal peel to a honey yellow or cinnamon buff. It is seldom as dark as is often found in the case of definite pits.

BROWNING OF THE OIL GLANDS

Another type of browning frequently observed is confined entirely to the oil glands. It is referred to in the present report as browning of the oil glands. Many glands in close proximity are affected, giving somewhat the effect of a mass discoloration, yet the tissue surrounding the oil glands usually remains normal in appearance (fig. 5). The color of the spots may range through various shades of brown, as described for scald.

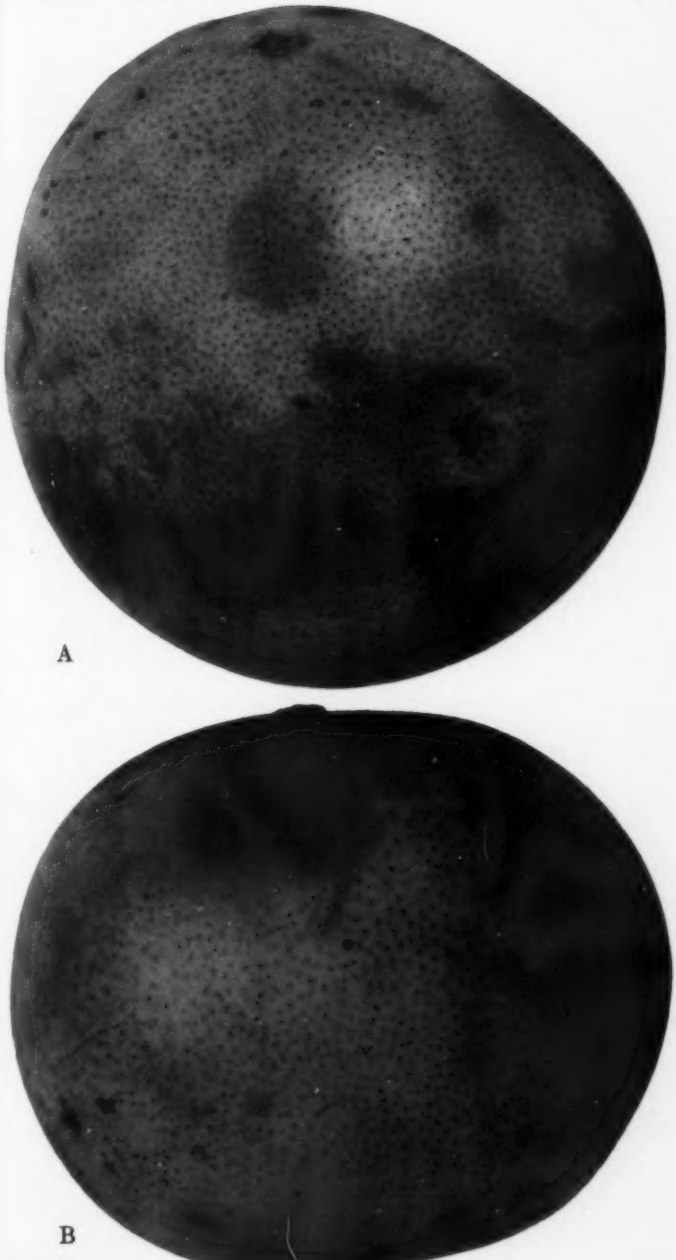
WATERY BREAK-DOWN

Watery break-down is a low-temperature disease that has been of rather common occurrence on fruit picked late in the season. The fruit becomes soft, spongy, and water-soaked, giving the appearance of having been frozen (pl. 1, *B*). The diseased condition may be particularly evident either in the peel or in the flesh of the fruit, but more often it is found in both. A study of a cross section of a grapefruit shows a general breaking down of the tissue. The carpels are loosely attached to the inner peel, and when a section of the peel is pressed a watery substance oozes freely from the albedo tissue. Tissue that has collapsed as a result of pits may be swollen so that the once pitted areas are no longer depressed. Affected fruits that are held for a short time at room temperature after removal from storage develop a disagreeable odor of fermentation.

ORGANISMS FOUND IN PITTED GRAPEFRUIT

In the spring of 1932 L. F. Butler, then associate pathologist, Division of Fruit and Vegetable Crops and Diseases, United States Department of Agriculture, began a study of the microorganisms found in the pitting lesions of grapefruit. Among a considerable number of organisms isolated, certain bacteria were found which appeared to be pathogenic. When these bacteria were forced into the

[3] Reference is made by number (italic) to Literature Cited, p. 351.



C. C. Krieger, Pinx. Lith. A. Hoen & Co.

A, Scald of heavily waxed grapefruit at end of 10 weeks' storage at 32° F. beginning April 30, 1934; *B*, watery break-down of heavily waxed grapefruit at end of 12 weeks' storage at 36° F. beginning February 12, 1934.

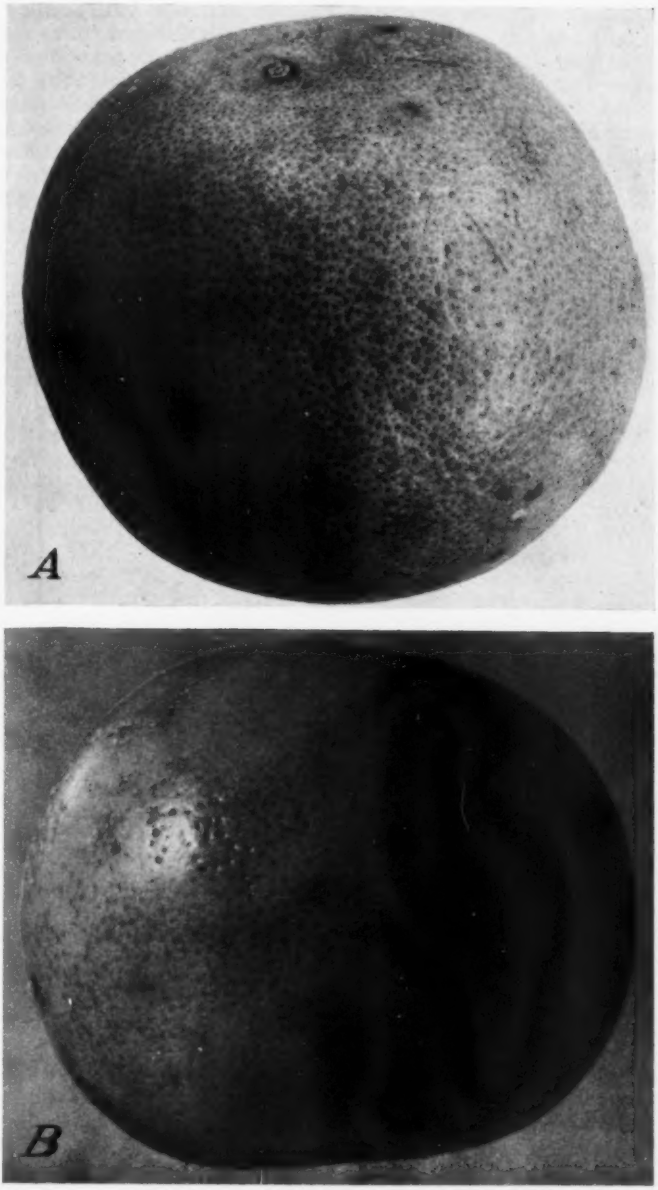


FIGURE 5.—Browning of oil glands on grapefruit (*A*) at end of 11 weeks' storage at 36° F. beginning May 7 1934, and (*B*) early stage, at end of 10 weeks' storage at 32° F. beginning Feb. 12, 1934.

peel by means of a hypodermic needle typical pits were produced and the organism could be recovered, whereas injections with sterile solutions failed to produce pits. Following Butler's resignation in June 1932 the further study of the problem was left with the present writers.

Butler found it difficult to obtain any organism from the pitted grapefruit tissue when it was transferred direct to agar, and adopted the method of first placing the tissue in sterile tap water and later transferring drops of the liquid to agar. This method was followed in the isolations reported herein; however, beef broth was often substituted for the sterile water. The usual method of making isolations was to remove the peel and transfer small pieces from the inner surface of the affected tissue direct to the liquid medium. The transfer from the liquid to the solid medium was made as soon as there was evidence of turbidity.

Transfers from normal-appearing albedo tissue (the white spongy portion of the peel) beneath the pits usually remained sterile, whereas transfers that included discolored tissue from the inner surface of the pits gave cultures of bacteria in 67 percent of 360 tests. No single organism predominated, but six or more were isolated repeatedly.

Bacteria were sometimes obtained from the tissue adjacent to the oil glands in normal peel.

The liquid cultures were held for several days after the transfer to solid media had been made, and were then examined for fungus as well as bacterial growth. Mycelium was seldom obtained and when present the fungus was usually found to be a species of *Penicillium*, although cultures of *Colletotrichum*, *Diplodia*, and *Phomopsis* were occasionally obtained (Florida grapefruit).

Microscopic studies were made of the pits in grapefruit that developed without inoculation and of those resulting from inoculations with organisms isolated from pitted tissue. Bacteria were present in large masses in the inoculated material, both in and around the oil glands (fig. 6). In the pits that had developed without inoculation bacteria were occasionally found but never in numbers that would support the conclusion they were the sole cause of the disease.

BACTERIA ISOLATED

In the separation and grouping of the various bacteria that have been isolated from pitted tissue considerable attention has been given to their growth characteristics. It will probably be sufficient in this connection to report some of the characteristics of the species or strain that has proved most virulent, originally isolated by Butler, and carried in culture as no. 38.

CULTURE NO. 38

Characters.—Motile by 2 to 5 peritrichous flagella; 0.84μ by 1.2μ to 2.5μ; capsules (72 hours); Gram-negative; colonies on agar smooth, glistening, yellow in center with gray margins; gelatin liquefied, napiform; in nutrient broth clouding medium, odor definite, pellicle enlarging with age and falling to bottom; nitrates reduced to nitrites, no gas; litmus milk slightly acidified, casein precipitated with extrusion of whey.

This description definitely separates the organism from *Bacterium citriputeale* C. O. Smith (*13*), the cause of black pit of lemons in California, and from *Pseudomonas citri* Hasse (*6*), the cause of citrus canker, but it leaves some question as to its relation to *Bacillus citrimaculans* Doidge, the cause of a spotting of citrus in South Africa (*3*).

The present organism is similar to *B. citrimaculans* in most respects but differs from it in the number of flagella and in the reaction to nitrates and to Gram's stain. The writers have not had the opportunity of comparing the two organisms in culture.

The organism (no. 38) has varied in size with growth conditions. It grew well on potato-yeast agar, and on a 24-hour culture of this medium it averaged 0.84μ by 2.0μ (fig. 7). When examined in grapefruit 8 days after inoculation it averaged 0.8μ by 1.0μ. After 1 or more week's growth in sterilized soil or sterilized tap water its

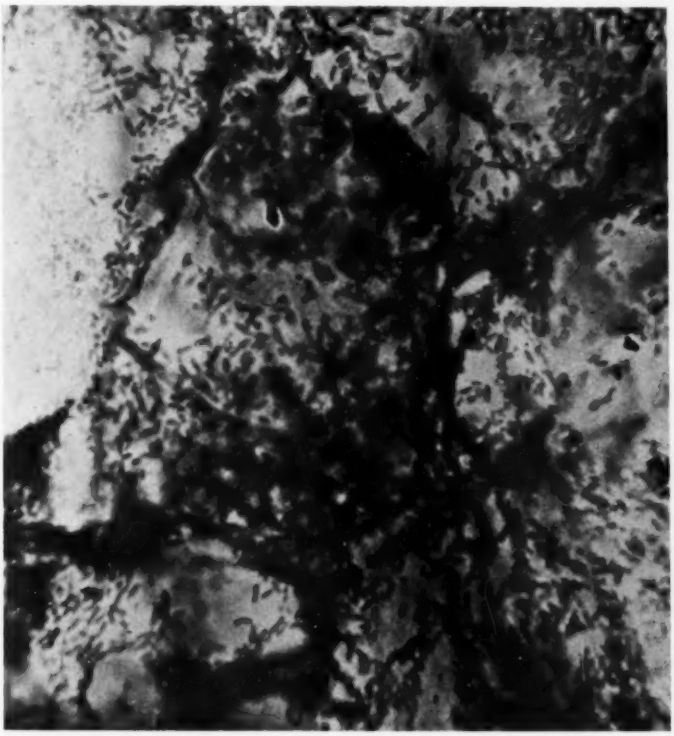


FIGURE 6.—Bacteria in grapefruit tissues after inoculation with organism no. 38 and storage at 40° F. for 13 weeks. × 1,440.

size was greatly reduced, but upon transfer to a more favorable medium it regained its former size.

The optimum temperature for the growth of the organism was between 75° and 80° F., and at temperatures of 32° and 40° the growth was extremely slow. This temperature response is not in harmony with the temperature conditions under which pitting and other blemishes of grapefruit are most prevalent (p. 324).

The organism has the power of breaking down lemon oil and oil obtained by steam distillation of grapefruit peel. When a layer of either of these oils was floated on top of a beef-broth culture at room

temperature it soon began to darken and finally broke down into hardened brown particles, whereas a similar layer of oil on uninoculated broth retained its normal character and appearance. Experiments were made in which the oil-destroying activity of culture no. 38 was compared with that of 20 other cultures, representing the complete range of bacterial organisms that had been isolated from grapefruit peel. In all cases a correlation was found between the oil-destroying and pit-producing powers. It has been pointed out previously (p. 326)

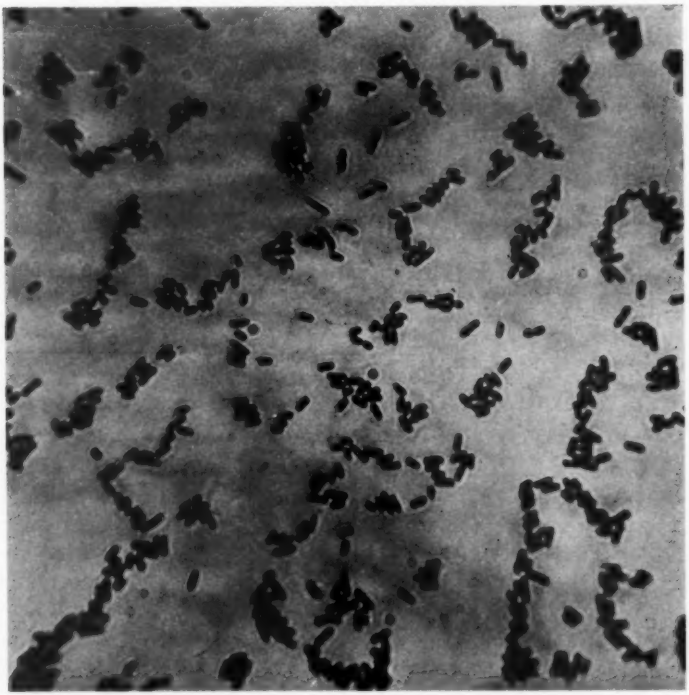


FIGURE 7.—Bacteria (culture no. 38) from a 24-hour culture on potato-yeast agar. × 1,440.

that bacteria were found in abundance both in and around the oil glands in pits that had been produced by inoculation.

The presence of bacteria, both in and around the oil glands, and the close correlation between their ability to produce pits and their power of breaking down oil suggests the possibility that the harmful effects of the bacteria may be due to their action upon the oil glands. Bates (*1*) has recently called attention to the fact that the oil glands of citrus furnish an avenue of infection for decay organisms.

INOCULATIONS

Inoculations with pure cultures of the bacteria isolated were made by means of a hypodermic needle. About 0.5 cc of liquid was used in each case. The needle was inserted tangentially and so that the

fluid would be released near the surface of the peel. The inoculum consisted of a heavy suspension of bacteria prepared in sterile water. Control injections with sterile water were made at the same time.

After the fruit had been held for a week to 10 days at 60° or 70° F. or for 5 to 7 weeks at 40°, brown spots 0.5 to 0.8 inch in diameter began to appear at the points where certain bacteria had been released (fig. 8). The spots gradually became darker and more sunken. Isolations made from these spots or pits soon after their appearance usually resulted in pure cultures of the original organism, but when isolations were delayed other bacteria were also obtained.

Bacteria from at least 20 different original isolations gave positive results when injected into the peel, but in spite of considerable variation in virulence and in growth the bacteria from these 20 cultures could probably be grouped under 2 or 3 species. No other organism

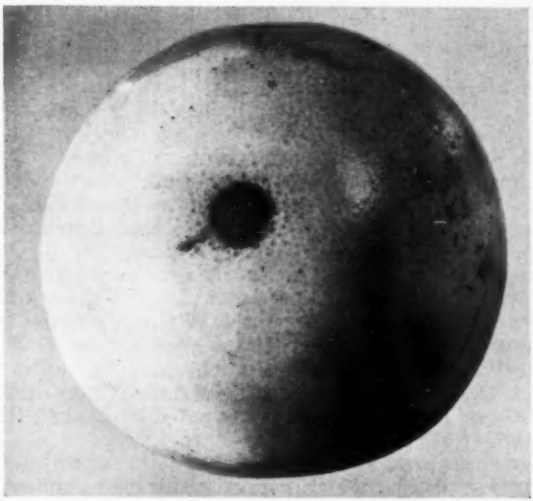


FIGURE 8.—Pit on grapefruit, produced by inoculation with bacteria (culture no. 38), after 7 weeks' storage at 40° F.

has given as strong positive results as that originally isolated by Butler and carried in culture as no. 38.

Injections with sterile water produced no injury when the fruit was held at 70° F., nor at 60°, but in later experiments part of the fruit was held at 40°, and it was found that after 5 to 7 weeks at this temperature definite pitting resulted from the sterile injections in about 50 percent of the tests as compared with 100 percent in similar injections that carried bacterial inoculum. Bacteria were isolated from some of the pits resulting from sterile injections, but in no case did they prove pathogenic when inoculated into the fruit.

Attempts were made to secure invasion by the isolated bacteria without wounding the peel. Grapefruits were immersed in bacterial suspensions and placed under a partial vacuum, with the idea that the return to normal pressure might force some of the bacteria into the peel. Other lots were heated to 100° F. for 1 day or desiccated

for several days and then bathed with bacterial suspensions. None of these treatments caused any increase in pitting.

In experiments reported later (p. 334), in which fruit was obtained direct from the trees, about a tablespoonful of soil from beneath the trees was placed inside each wrapper as the fruit was being packed. After 10 weeks' storage at 32° F. the fruit packed in this manner was found to have several times as much pitting as control fruit packed without the soil. The results led to the use of soil that had been sterilized and then inoculated with the bacteria isolated from grapefruit. The soil was examined before using and was known to have an abundant growth of bacteria. After several weeks in storage the soil-coated fruit had far more pitting than fruit packed in the usual manner. But it was found that when sterile soil was used the increase in pitting was fully as great as with the inoculated soil. The results of these experiments will be discussed further under the heading Humidity and Desiccation (p. 336.)

Significance

The presence of bacteria in a considerable percentage of the pitted tissue and the fact that pits were produced by inoculation with the isolated organisms and the bacteria recovered might be taken as conclusive proof that the disease is of bacterial origin, but there are points in opposition to this simple explanation. Bacteria were found in abundance in pits that were produced by inoculation, but were difficult to locate, if present at all, in pits that developed without inoculation. Although bacteria were obtained in 67 percent of the isolations, less than 10 percent of the cultures were found capable of producing typical pits. At temperatures at which the disease usually occurs pits resulted from sterile as well as from bacterial injections.

Temperature studies showed that the growth rate of the bacteria was extremely slow at 32° to 40° F. and gradually increased up to 75° or 80°, whereas, in studies that follow, it is shown that pitting is worst at 32° to 40° and is almost entirely prevented by holding the fruit at a temperature of 50° or higher.

In view of these facts and others that are developed later showing the sensitiveness of the grapefruit peel to desiccation and to various types of injury, it seems necessary to conclude that while bacteria may sometimes serve as a contributing and modifying factor in the development of pitting, they cannot be considered the fundamental cause of the disease.

The following storage experiments point out some of the factors involved in the development of pitting and other grapefruit diseases.

STORAGE EXPERIMENTS

MATERIAL AND METHODS

The origin and the packing-house treatment of the fruit used in the various storage experiments are shown in table 1. The different lots will be referred to later by number.

TABLE 1.—*Description of the different lots of grapefruit used in experimental storage*

Lot no.	Size[1]	Date of experiment	Origin	Where purchased [2]	Packing-house treatment	Notes
		1931				
1A	64	Oct. 27	Orlando, Fla.	Orlando, Fla.	None	Experiments started after shipment to Washington, D. C., by ordinary express.
1B	64	Nov. 27	do	do	do	Do.
1C	64	Dec. 30	do	do	do	Do.
		1932				
1D	64	Jan. 29	do	do	do	Do.
1E	64	Feb. 10	do	do	do	Do.
1F	64	Feb. 26	do	do	do	Do.
2	64	Apr. 13	do	do	do	Experiments started before shipment to Washington, D. C., by ordinary express.
3	64	Apr. 16	do	do	do	Do.
4	70	Oct. 19	Cuba	Washington, D. C.	Unknown	
5	80	Nov. 9	Vero Beach, Fla.	do	do	
6	80	Nov. 21	Unknown	do	do	
7	80	Dec. 7	do	do	do	
		1933				
8	80	Jan. 13	Largo, Fla.	do	do	
9A	64	Feb. 11	Brooksville, Fla.	Brooksville, Fla.	None	Experiments started after shipment to Washington, D. C., by ordinary express.
9B	64	do	do	do	Colored and waxed.	Do.
10	80	Mar. 9	Palmetto, Fla.	Washington, D. C.	Waxed and probably colored.	
11	80	Mar. 10	Vero Beach, Fla.	do	Unknown	
12	80	Mar. 31	do	do	do	
13	80	Apr. 11	Auburndale, Fla.	do	Waxed and probably colored.	
14	70	Apr. 14	Orlando, Fla.	do	None	Hauled in bulk by truck from Orlando to Washington, D.C.
15	64	Apr. 21	Largo, Fla.	do	Unknown	
16	70	Apr. 28	Orlando, Fla.	do	None	Do.
17	70	May 5	Lake Wales, Fla.	do	Waxed and probably colored.	
18	64	Nov. 16	Brooksville, Fla.	Brooksville, Fla.	Colored but not waxed.	Experiment started after shipment to Washington, D. C., by ordinary express.
19	64	Nov. 27	do	do	do	Do.
20	64	Dec. 19	do	do	do	Do.
		1934				
21	70	Feb. 12	Vero Beach, Fla.	Vero Beach, Fla.	do	Do.
22	64	Mar. 10	Orlando, Fla.	Orlando, Fla.	do	Do.
23	64	Apr. 19	do	do	None	Do.
24	64	Apr. 30	do	do	do	Do.
25	70	May 7	do	do	do	Do.

[1] Number of grapefruits per box.

[2] The writers are indebted to John R. Winston for cooperation in securing the grapefruit purchased in Florida.

Small lots of fruit were used, but great care was taken to have the different lots uniform. Individual notes were taken on each fruit, and the degree of scald and pitting was recorded on the basis of actual area affected. These individual records were combined and converted into percentages of the total fruit areas. Notes were usually taken at intervals of 2 to 5 weeks, and a number of the lots were held at outside temperature for 1 week after storage before the final note-taking. Unless otherwise stated, the results reported are based on the condition of the fruit while in storage or at the time of removal from storage.

FACTORS AFFECTING STORAGE DISEASES

TEMPERATURE

Pitting has been generally recognized as a low-temperature disease, and storage at 45° F. or higher has been recommended as a means of control (*4*, *5*, *8*, *9*, *12*). Friend and Bach (*5*) found that scald is similarly affected by low temperature. The results of the present studies are in harmony with these conclusions. The development of pitting at different storage temperatures is shown in table 2.

TABLE 2.—*Effect of storage temperature* [1] *on pitting of grapefruit*

Lot no.	Fruits under each condition	Storage period	Peel showing—													
			Definite pitting at indicated temperature							Mild pitting at indicated temperature						
			30	32	34	36	40	50	60	30	32	34	36	40	50	60
	No.	*Weeks*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*
4	10	7	-----	-----	-----	-----	1.59	0.05	-----	-----	-----	-----	-----	1.10	0.01	-----
5	8	10	-----	-----	-----	-----	.25	0	-----	-----	-----	-----	-----	.46	.01	-----
9B	8	8	-----	0.31	-----	4.00	2.80	-----	-----	-----	1.60	-----	0.34	1.00	-----	-----
11	8	14	-----	1.90	-----	10.70	9.00	-----	0.09	-----	.45	-----	1.40	3.20	-----	0
13	8	13	-----	.07	-----	-----	-----	0	0	-----	0	-----	-----	-----	.01	.02
14	8	10	-----	1.30	-----	-----	.74	.50	-----	-----	1.20	-----	-----	0	0	-----
15	9	13	-----	.58	-----	1.40	-----	.01	-----	-----	.09	-----	.34	-----	0	-----
16	8	12	-----	.88	-----	3.60	-----	.28	-----	-----	.40	-----	.17	-----	0	-----
17	9	10	-----	.40	-----	3.80	-----	.06	-----	-----	.13	-----	.30	-----	0	-----
18	24	8	-----	.005	-----	-----	.067	.028	-----	-----	.44	-----	-----	.058	.042	-----
19	16	15	-----	.18	-----	.71	-----	.09	-----	-----	.41	-----	.10	-----	0	-----
20	24	13	-----	.30	-----	.50	1.00	0	-----	-----	.30	-----	.13	.56	0	-----
21	16	8	-----	.04	-----	2.00	1.45	.031	-----	-----	.03	-----	.98	.56	0	-----
22	16	9	-----	.49	0.67	.66	1.50	.51	-----	-----	1.60	1.10	.30	1.90	.26	-----
23	14	8	0.06	.02	.95	.10	-----	0	-----	0.08	.01	.19	.01	-----	0	-----
24	24	10	-----	.23	.54	.25	-----	0	-----	-----	.39	.43	.10	-----	.02	-----
25	24	10	0	.06	3.70	3.10	1.90	0	-----	.003	1.80	.65	.72	.24	.003	-----

[1] All lots with the exception of those at 34° F. were held at a relative humidity of 85 to 90 percent. The 34° lots were held in commercial storage.

After 8 or more weeks in storage there was more definite pitting at 36° F. than at 32° in all of the 12 cases in which these two temperatures were compared, and the average pitting was more than six times as great at the higher temperature (table 2). In a number of the experiments (lots 11, 15, 16, and 17) sufficiently frequent notes were taken to follow the gradual progress of pit development at the two temperatures. The results are shown in figure 9. It will be seen that the above proportion was well maintained throughout the storage period.

In contrast with the foregoing results there was more mild pitting at 32° F. than at 36° in 7 of the 12 tests reported in table 2, and an

average in the 12 tests shows nearly twice as much of this type of pitting at the lower temperature.

When fruit was held at an outside temperature of about 70° F. for 1 week after removal from storage, it was found that there was much greater increase in both forms of pitting on the fruit from 32° storage than on that from 36°. The average for 6 different lots (nos. 19, 21, 22, 23, 24, and 25) showed about twice as much definite pitting on the fruit from 36° storage as on that from 32° and nearly three times as much mild pitting on the fruit from 32° storage as on that from 36°. The final contrast was thus largely in type of pitting with little difference in area of peel affected. The apparently better results at 32° seem to have been due largely to a greater delay in the expression of a condition rather than to a difference in area of peel affected.

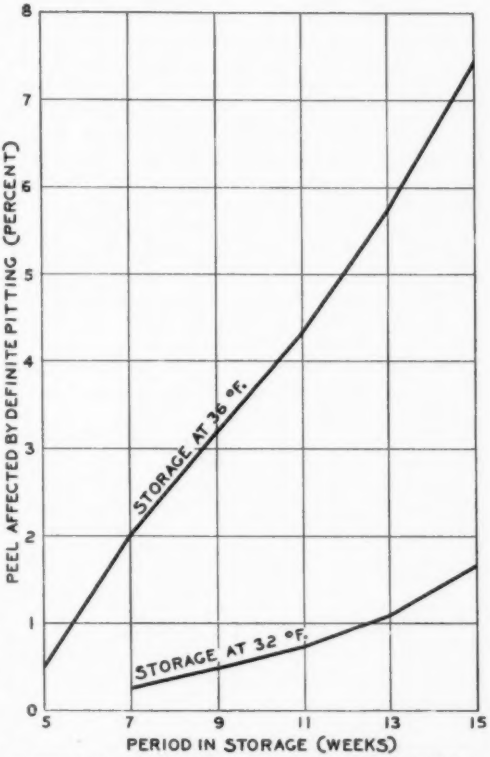


FIGURE 9.—Development of definite pitting on grapefruit held in storage at 32° F., compared with that on grapefruit held in storage at 36°.

The data of table 2 do not justify the conclusion that there was much difference between the pitting at 40° F. and that at 36°, nor between the pitting at 30° and that at 32°. The pitting at 34° was somewhat greater than that at 36°, but the 34° lots were in commercial storage and the condition of the fruit upon removal indicated the probability that the humidity had been lower than with the other lots. Upon removal from storage the increase in pitting on the fruit from 34° and 40° was similar to that on the fruit from 36°, and the increase at 30° similar to that at 32°.

The big contrast, and the significant one that is shown by table 2, is the low percentage of pitting at 50° and 60° F., as compared with that at the lower temperatures. This contrast became still greater after removal from storage. Whereas the percentage of pitting on the fruit from 36° and 40° storage often doubled and that on the fruit

from 32° storage often quadrupled within a week, the fruit from 50° and 60° storage showed little or no increase, remaining practically free from all forms of pitting. It has been previously pointed out (p. 329) that injections with sterile water which sometimes caused injury at 40° failed to produce injury at 60° and 70°.

In the later experiments (lots 19 to 25, inclusive) a definite record on oleocellosis was kept. Oleocellosis was of very erratic and rather rare occurrence, and its prevalence apparently was greatly influenced by mechanical injury and packing conditions; but it should be noted that there was occasional occurrence at temperatures of 32° to 40° F., whereas the fruit at 50° remained practically free from the disease.

In the seven experiments with the 1933–34 crop careful records were kept on the browning of the oil glands, scald, and watery break-down. All of these diseases were of erratic occurrence, the susceptibility of the fruit apparently increasing with maturity. All were decidedly more common at 32° than at 36° F. None occurred at 50° (standard packing), and scald and watery break-down were never found at 40°.

Storage temperatures of 50° and 60° F., while preventing pitting and other physiological disorders, favored the development of decay. After 8 weeks at 50° or higher the fruit showed 12 to 60 percent, and averaged about 30 percent, of stem-end rot, whereas the fruit held at 40° or lower usually showed no decay. After longer storage periods there was occasional blue or green mold decay at the lower temperatures. When the fruit was held continuously in storage there was little or no contrast between the decay at 32° and at 36°, but when held for 1 week at room temperature after removal from storage there was about twice as much decay on the fruit from 36° as on that from 32° storage.

After short storage periods the fruit held at 50° or 60° F. seemed to have somewhat the best flavor, but later it became distinctly flat as compared with fruit held at 40° or lower. The fruit held at 36° or 40° seemed in some instances to be superior to that held at 32°.

Humidity and Desiccation

In a number of the experiments fruit was stored under two different humidities, namely 85 to 90 percent (table 2) and 65 to 75 percent. The comparative results from the two storage conditions are shown in table 3.

In 4 of the 6 tests of storage at 40° F., mild pitting was greater with low humidity than with high humidity and averaged in the 6 tests about 41 percent greater. In all 6 of the same tests definite pitting was greater with low than with high humidity and averaged nearly five times as great. In the 1 test at 50° F. there was not enough pitting with either high or low humidity to be of any great significance. In all of the tests the fruit held at low humidity was more shriveled and had greater loss of weight than that held at high humidity.

It was pointed out on page 330 that placing about a tablespoonful of orchard soil inside each fruit wrapper increased pitting. The results of this and similar treatments are reported in table 4.

The addition of the soil increased definite pitting in 16 and mild pitting in 14 out of a total of 19 tests. An average of the results shows that the soil inclusions caused an increase of approximately 100 percent in both forms of pitting. The increase was as great at 50° F.

as at the lower temperatures. It was somewhat greater with low humidity than with high humidity and fully as great with sterile soil as with inoculated soil.

TABLE 3.—*Effect of humidity on pitting of grapefruit stored at 40° and 50° F.*

Lot no.	Fruits under each condition	Storage period	Storage temperature	Peel showing—			
				Definite pitting		Mild pitting	
				With low humidity [1]	With high humidity [2]	With low humidity [1]	With high humidity [2]
	Number	*Weeks*	*°F.*	*Percent*	*Percent*	*Percent*	*Percent*
4	10	7	40	2.19	1.59	1.92	1.10
4	10	7	50	.04	.05	.05	.01
5	8	10	40	.97	.25	.63	.46
9B	8	8	40	13.20	2.80	1.40	1.00
10	8	4	40	1.10	.15	0	.40
11	8	4	40	.82	.35	0	.06
14	8	10	40	10.00	.74	.22	0

[1] Relative humidity 65 to 75 percent.
[2] Relative humidity 85 to 90 percent.

TABLE 4.—*Pitting of grapefruit as affected by including soil, sand, or charcoal within the wrapper*

Lot no.	Fruits under each condition	Storage temperature	Storage humidity [1]	Storage period	Foreign matter included	Peel showing—			
						Definite pitting		Mild pitting	
						With inclusion	Without inclusion	With inclusion	Without inclusion
	Number	*°F.*		*Weeks*		*Percent*	*Percent*	*Percent*	*Percent*
3	35	32	High	6	Grove soil	0.95	0.22	0.24	0.007
4	10	40	do	7	Inoculated soil	1.59	1.15	1.10	.27
	10	40	Low	7	do	7.36	2.19	2.87	1.92
	10	50	High	7	do	.55	.05	0	.01
	10	50	Low	7	do	2.38	.04	1.53	.05
5	8	40	High	10	do	1.60	.25	1.97	.46
	8	40	Low	10	do	7.50	.97	12.80	.63
	8	50	High	10	do	.13	0	0	.01
7	20	40	Low	6	do	3.80	1.34	4.45	1.84
	20	40	do	6	Sterile soil	5.47	1.34	5.15	1.84
8	6	36	High	6	Inoculated soil	2.10	1.90	.40	.87
	6	36	do	6	Sterile soil	.62	1.90	.65	.87
	6	40	Low	6	Inoculated soil	2.84	1.00	3.85	1.20
	6	40	do	6	Sterile soil	2.68	1.00	2.68	1.20
9A	8	36	High	8	Inoculated soil	7.20	9.70	1.30	1.70
	8	40	do	8	do	2.60	5.10	.40	.17
	8	40	do	8	Sterile soil	6.00	5.10	.37	.17
	8	40	Low	8	Inoculated soil	5.20	3.80	1.10	.74
	8	40	do	8	Sterile soil	9.00	3.80	3.90	.74
	8	40	do	8	Quartz sand	9.30	3.80	6.50	.74
13	8	40	do	10	Animal charcoal	.50	.19	0	0
	8	40	do	10	Vegetable charcoal	.85	.19	0	0

[1] The low-humidity rooms were held at 65 to 75 percent relative humidity and the high humidity rooms at 85 to 90 percent.

It seemed possible that the increase in pitting might be due to some harmful substance in the soil; quartz sand and vegetable and animal charcoal were therefore tried, but with results similar to those obtained with soil.

The fruit packed with foreign material inside the wrapper usually showed extreme shriveling and loss of weight; this was greater when the foreign material was applied as a moist smear than when held loose in the wrapper. Pitting showed a similar contrast. The shriveling was worse when the fruit was held at low than at high humidity; a similar contrast has been pointed out for pitting, both with and without the presence of foreign material.

In some instances the soil smear was washed off after 5 to 9 days and the fruit was then wrapped in oiled paper, with the result that shriveling and pitting were only very slightly increased by the soil treatment.

In other experiments (lots 21, 22, and 23) glycerin wrappers were used on the fruit in comparison with ordinary wrappers. The results are shown in table 5. The addition of the glycerin increased definite pitting to an average of about 7 times and mild pitting to an average of almost 3 times that with ordinary wrappers. There was an increase in definite pitting at 50° F. as well as at the lower temperatures. There was a relatively high percentage of mild pitting on the treated fruit at both 36° and 32°, whereas definite pitting was much more severe at 36° than at 32°. The fruit packed in glycerin wrappers showed more shriveling and much greater loss in weight than the fruit in untreated wrappers.

TABLE 5.—*Effect of glycerin wrappers* [1] *on pitting of grapefruit at different storage temperatures*

Lot no.	Fruits under each condition	Storage period	Storage temperature	Peel showing—			
				Definite pitting		Mild pitting	
				With glycerin wrapper	With ordinary wrapper	With glycerin wrapper	With ordinary wrapper
	Number	*Weeks*	*° F.*	*Percent*	*Percent*	*Percent*	*Percent*
21	16	8	32	0.32	0.04	3.00	0.03
21	16	8	36	12.00	2.00	3.50	.98
22	16	9	32	.07	.49	.13	1.59
22	16	9	36	7.10	.66	1.50	.30
23	14	8	36	2.80	.10	.22	.01
23	14	8	50	.32	0	0	0

[1] In lot 22 the wrappers were saturated with glycerin before using, but in lots 21 and 23 glycerin was rubbed on the fruits and dry wrappers then applied. The glycerin was taken up freely by the wrappers.

In the experiments with soil and glycerin the correlation between shriveling and the development of pitting was so close that it seems necessary to consider the drying effects associated with the use of these materials as a factor in the production of the disease. Whether this drying was brought about largely by direct absorption of water or more indirectly by action upon the oil or other peel constituents has not been determined.

There was no increase in the browning of the oil glands, scald, or watery break-down resulting from the use of glycerin wrappers nor from the inclusion of soil within the wrappers.

DELAYED STORAGE, HIGH TEMPERATURE, AND TEMPERATURE CHANGES

The sharp decrease in pitting and the accompanying increase in decay at 50° F. as compared with 40° and lower suggested the importance of determining whether similar control of the disease could

be obtained without continued exposure to higher temperatures. With this point in mind three types of experiments were planned: Delayed storage at 50° or higher, exposure to relatively high temperatures before storing, and removal from low-temperature storage at stated intervals.

Hawkins (*7*) and Hawkins and Barger (*8*) recommended curing grapefruit before placing it in storage, either by delaying it for a week to 10 days at 70° to 75° F., with a humidity of about 65 percent, or by treating it with gas from a kerosene stove in a coloring room for 3 to 4 days. Both treatments decreased pitting in storage, but the latter method was considered the better because of the lower percentage of decay. Friend and Bach (*5*) have reported that scald as well as pitting was reduced by holding the fruit at 70° for 10 days before storing.

In the present experiments the period of delayed storage at 60° F. or above was not extended beyond 3 to 5 days, yet it resulted in a definite reduction in pitting. In the four tests reported in table 6 there was an average of about three times as much pitting on fruit that was stored immediately as on that delayed at 60° or above. It should be noted, however, that all lots of fruit used in these tests were purchased on the Washington market and must have been picked several days before the experiment was begun.

In other experiments the fruit was held at 50° F. during the delay. In 3 lots the fruit was held at this temperature for 2 days, in 4 lots for 7 to 10 days, and in 8 lots for 14 or 15 days, before being stored at 32° or 36°. The data in table 6 do not indicate any consistent benefit from these delays. The results from a delay of 7 to 10 days at the higher temperature before storage at 32° and 36° were more favorable than those from a delay of 2 days or 2 weeks.

TABLE 6.—*Effect of delayed storage at 50° F. and above on pitting of grapefruit stored later at 32° and 36°*

Lot no.	Fruits under each condition	Storage period	Storage temperature	Period of delay	Temperature during delay	Peel showing—			
						Definite pitting		Mild pitting	
						With delay	With immediate storage	With delay	With immediate storage
	Number	*Weeks*	*°F.*	*Days*	*°F.*	*Percent*	*Percent*	*Percent*	*Percent*
8	8	6	36	4	60	0.28	1.00	0.01	1.20
8 [1]	8	6	36	4	60	.05	.27	.03	0
11	8	7	36	3	70–75	1.25	4.00	.22	.80
15	9	13	36	5	70–75	1.10	1.40	.20	.34
18	8	12	32	2	50	.13	.53	.25	.25
18	8	12	32	7	50	.17	.53	.37	.25
18	8	12	32	14	50	.07	.53	.13	.25
19	8	15	32	2	50	.35	.18	.32	.41
19	8	15	32	10	50	.11	.18	0	.41
19	8	15	32	15	50	.77	.18	.06	.41
19	8	15	36	2	50	1.70	.71	.08	.10
19	8	15	36	15	50	3.70	.71	.07	.10
20	16	13	32	7	50	.35	.30	.17	.30
20	16	13	32	14	50	.35	.30	.15	.30
20	16	13	36	7	50	.22	.50	.40	.13
20	16	13	36	14	50	1.85	.50	.10	.13
21	16	10	32	14	50	.12	.07	.12	.08
21	16	10	36	14	50	1.30	3.30	1.00	2.00
21 [2]	16	10	32	14	50	.04	.02	0	.007

[1] In oiled wrappers.
[2] Fruits coated with wax.

In other experiments the prestorage period at the higher temperatures was shortened to less than 24 hours and the fruit was held in an incubator at a temperature usually slightly above 100° F. (table 7). Of the 12 instances in which the fruit was stored at 36° or 40° after exposure to high temperature there were 11 in which definite pitting was decreased, and the average definite pitting for the 12 heated lots was less than one-fifth of that for the fruit stored immediately. The one instance in which definite pitting was not decreased was with fruit heated but 6 hours. Mild pitting was decreased in 6 out of the 8 cases where it was present in the control, and the average mild pitting for the 8 heated lots was about one-third of that for the fruit stored immediately. Of the 11 instances in which the fruit was stored at 32° there was an average of about twice as much definite pitting but less than two-thirds as much mild pitting on the heated fruit as on that which was stored immediately.

TABLE 7.—*Effect of prestorage heating at temperatures of 98° F. and above on development of pitting and scald on grapefruit stored at 32° to 40°*

Lot no.	Fruits under each condition	Storage period	Storage temperature	Prestorage period of heating	Temperature during heating	Peel showing—					
						Definite pitting		Mild pitting		Scald [1]	
						With heating	Without heating	With heating	Without heating	With heating	Without heating
	Number	*Weeks*	*°F.*	*Hours*	*°F.*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
9A	7	8	[2] 40	20	109	0.26	3.80	0	0.74	0	0
9B	7	8	[2] 40	20	109	.44	13.20	.02	1.40	0	0
13	8	10	32	6	102	.70	.04	.18	.50	.37	0
13	8	10	32	22	105	.09	.04	0	.50	4.60	0
13	8	10	[2] 40	6	102	.50	.19	0	0	0	0
13	8	10	[2] 40	22	105	.12	.19	0	0	0	0
14	8	10	32	20	100	3.40	1.30	1.80	1.20	0	0
14	8	10	[2] 40	20	100	3.00	10.00	.08	.22	0	0
15	9	9	32	17	104	.03	.11	0	.17	7.00	0
15	9	9	36	6	102	.20	.78	.22	.10	0	0
15	9	9	36	17	104	.49	.78	0	.10	7.80	0
16	8	8	32	19	110	.31	.30	0	0	6.30	3.50
16	8	8	36	6	102	.32	2.80	.07	0	0	0
16	8	8	36	19	110	.77	2.80	0	0	0	0
17	9	10	32	6	108	.20	.40	.10	.13	1.20	2.30
17	9	10	32	20	100	.35	.40	0	.13	12.20	2.30
17	9	10	36	20	100	.15	3.80	.64	.30	0	0
19	8	15	32	21	100	.87	.18	.33	.41	0	0
19	8	15	[3] 32	21	100	.20	.18	.10	.41	3	0
19	8	15	36	21	100	.30	.71	.03	.10	0	0
20	16	13	32	20	98	.74	.30	.04	.30	0	0
20	16	13	[4] 32	20	98	.03	.30	0	.30	0	0
20	16	13	36	20	98	.32	.50	.05	.13	0	0

[1] Much of the scald bordered upon a watery break-down condition.
[2] With low humidity.
[3] Held at 50° F. for 10 days after heating.
[4] Held at 50° F. for 6 days after heating.

The effect of heating and the modifying influence of storage temperature were still more pronounced in the case of scald. Of the fruit that was stored at 32° F. there were 2 lots in which scald was greatly increased by heating and 2 lots in which a high percentage of scald developed on the heated fruit, while the fruit that was stored immediately remained free from the disease. In contrast with this there was only 1 lot in which scald occurred on the heated fruit at 36° and none in which it was found on the fruit that was stored immediately at that temperature. Much of the scald that resulted from heating bordered upon watery break-down. It should be noted that

all of the lots in which scald occurred were picked in April or later. (See table 1.)

In other experiments the fruit was removed from storage to higher temperatures (table 8). At 40°, 50°, and 60° F. the temperature was carefully controlled. The 70° temperature was only approximate, ranging from 65° to 75°.

Of the 15 tests in which fruit was removed from storage at 32° or 36° F. to 70° for 1 day at the end of 1 week and again at the end of 2 weeks (table 8, unwaxed grapefruit), there were 13 cases in which there was less definite pitting and 12 cases in which there was less mild pitting on the fruit that had been moved than on that which remained continuously in storage. The average of the results shows that both forms of pitting were reduced to less than one-fourth of that found on the controls.

TABLE 8.—*Effect of temperature changes on storage diseases of unwaxed and of heavily waxed grapefruit*

UNWAXED GRAPEFRUIT

Lot no.	Fruits under each condition	Storage period	Storage temperature	Fruits moved to indicated temperature (° F.)	Peel showing—								Fruits showing watery breakdown	
					Definite pitting		Mild pitting		Browning of oil glands		Scald			
					With moving	Without moving	With moving	Without moving	With moving	Without moving	With moving	Without moving	With moving	Without moving
	No.	*Weeks*	*° F.*		*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
8	8	6	36	70° for 1 day after 1 week and again after 2 weeks of storage	0.57	1.90	0.02	0.87						
9A	4	8	36	do	.51	9.70	0	1.70						
9B	4	8	36	do	.48	4.00	.16	.34						
15	9	14	32	do	.20	.58	.01	.09						
15	9	14	36	do	.29	1.40	.15	.34						
16	8	8	32	do	.97	.30	0	3.50						
16	8	8	36	do	.07	2.80	.13	0						
18	8	12	32	do	.13	.53	.07	.25						
19	8	12	32	do	.12	.46	.50	1.45						
20	8	13	32	do	.04	.30	0	.30	0.05	4.50				
20	8	13	36	do	.52	.50	.67	.13	.12	.05				
21	16	8	32	do	.004	.04	.04	.03	0	0	0	0	0	0
21	16	8	36	do	.90	2.00	.65	.98	0	0	0	0	0	0
22	16	9	32	do	.03	.49	.05	1.60	0	0	0	0	0	0
22	16	9	36	do	.51	.66	.08	.30	0	0	0	0	0	0
23	14	8	32	70° for 2 days after 1 week's storage	.01	.02	.06	.01	0	0	0	.85	0	0
25	16	10	32	do	0	.06	.004	1.80	0	0	0	1.30	6.3	6.3
22	16	9	32	70° for 1 day after 2 weeks' storage	.04	.49	.12	1.60	0	0	0	0	0	0
23	14	8	32	70° for 2 days after 2 weeks' storage	.004	.02		.01	0	0	0	.85	0	0
25	16	10	32	60° for 2 days after 2 weeks' storage	.004	.06	.15	1.80	0	0	.21	1.30	12.5	6.3
25	16	10	34	60° for 2 days after 2 weeks' storage	.56	3.70	.40	.65	0	0	0	0	0	18.8
25	16	10	36	do	.52	3.10	.22	.72	0	0	0	0	12.5	18.8
23	14	8	32	70° for 2 days after 3 weeks' storage	0	.02	.08	.01	.12	0	.27	.85	0	0
25	16	10	32	60° for 3 days after 3 weeks' storage	.17	.06	.05	1.80	0	0	0	1.30	0	6.3
25	16	10	32	60° for 3 days after 4 weeks' storage	.11	.06	.04	1.80	0	0	0	1.30	0	6.3
18	8	12	32	50° for 1 week after 2 weeks' storage	.06	.53	0	.25						
19	8	12	32	do	.15	.46	1.17	1.45						
20	8	13	32	do	.015	.30	0	.30	.07	4.50	0	0	0	0

TABLE 8.—*Effect of temperature changes on storage diseases of unwaxed and of heavily waxed grapefruit*—Continued

Lot no.	Fruits under each condition	Storage period	Storage temperature	Fruits moved to indicated temperature (° F.)	Peel showing—								Fruits showing watery breakdown	
					Definite pitting		Mild pitting		Browning of oil glands		Scald			
					With moving	Without moving	With moving	Without moving	With moving	Without moving	With moving	Without moving	With moving	Without moving
	No.	*Weeks*	*° F.*		*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
20	8	13	36	50° for 1 week after 2 weeks' storage	0.80	0.50	0.07	0.13	0	0.05	0	0	0	0
21	16	8	32	do	.10	.04	.04	.03	.04	0	0	0	0	0
21	16	8	36	do	.50	2.00	.20	.98	.02	0	0	0	0	0
22	16	9	32	do	.06	.49	1.30	1.60	0	0	0	0	0	0
22	16	9	36	do	.87	.66	.14	.30	0	0	0	0	0	0
23	14	8	32	do	.16	.02	.05	.01	0	0	0	.85	0	0
25	16	10	32	do	0	.06	.01	1.80	0	0	.60	1.30	0	6.3
25	16	10	34	do	.20	3.70	.10	.65	0	0	0	0	0	18.8
25	16	10	36	do	1.50	3.10	.20	.72	0	0	0	0	0	18.8
24	16	10	32	50° permanently after 1 week's storage	.02	.23	0	.39	0	1.10	0	0	0	6.3
24	16	10	32	50° permanently after 2 weeks' storage	.04	.23	0	.39	0	1.10	0	0	0	6.3
25	16	10	32	do	.04	.06	0	1.80	0	0	0	1.30	0	6.3
25	16	10	34	do	.02	3.70	0	.65	0	0	0	0	0	18.8
25	16	10	36	do	.08	3.10	0	.72	0	0	0	0	0	18.8
24	16	10	32	50° permanently after 3 weeks' storage	.10	.23	.03	.39	0	1.10	0	0	0	6.3
				HEAVILY WAXED GRAPEFRUIT										
18	8	12	32	70° for 1 day after 1 week, and again after 2 weeks of storage	0	0	0.13	23.20						
19	8	15	32	do	0	0.07	.32	2.11	0.32	1.10	0	0	0	0
20	8	13	32	do	.03	1.10	.01	.06	.20	9.20	0	0	0	0
20	8	13	36	do	.20	.34	.15	.05	0	0	0	0	0	0
21	16	10	32	do	0	0	.004	.04	0	0	0	0	6.3	25.0
22	16	9	32	do	.02	.22	.13	.55	0	0	0	0	0	0
22	16	9	32	70° for 1 day after 2 weeks' storage	.04	.22	.12	.55	0	0	0	0	0	0
23	14	8	32	70° for 2 days after 2 weeks' storage	.01	0	.03	.02	.50	0	0	12.1	0	4.8
21	16	10	32	50° for 1 week after 2 weeks' storage	.02	0	.01	.04	0	0	0	0	18.8	25.0
22	16	9	32	do	.003	.22	.08	.55	0	0	0	0	0	0
22	16	9	36	do	.20	.18	.01	.20	0	0	0	0	0	0
23	14	8	32	do	0	0	0	.02	0	0	8.3	12.1	0	4.8
24	16	10	32	50° permanently after 2 weeks' storage at 32°	.004	.02	0	.03	.18	.22	0	4.3	0	6.3

In the few tests in which the fruit was moved to 60° F. instead of 70° or moved to 60° or 70° only once by the end of 2 weeks' storage, the average results were fully as good as those reported above, but in the 3 cases in which the change in temperature was delayed to the end of 3 weeks or later there was little evidence that it caused any reduction in pitting.

In other experiments the grapefruit was removed from 32° or 36° F. at the end of 2 weeks' storage to 50° for 1 week and then returned to the original temperature (table 8, unwaxed grapefruit). The results were not as favorable as with the shorter periods of exposure to higher temperatures, yet out of the 12 tests there were 8 instances in which there was less definite pitting and 10 in which there was less mild pitting on the fruit that had been moved than on that which remained at

a uniform temperature. The average of the results shows that both forms of pitting on the moved fruit were reduced to approximately one-third of that found on the controls.

In other experiments the grapefruit was moved from 32°, 34°, or 36° F. to 50° permanently at the end of 1, 2, or 3 weeks (table 8, unwaxed grapefruit). In the 1 test in which the change was made at the end of 1 week and in the 4 tests in which it was made at the end of 2 weeks, mild pitting was entirely prevented and definite pitting reduced to an average of about 3 percent of that found on the controls. In the 1 instance in which the change was made at the end of 3 weeks, both forms of pitting were far less serious on the fruit that had been moved than on that which had remained continuously at the lower temperature.

With the heavily waxed fruit (table 8) there was usually much less pitting on the controls (at constant temperature), yet the percentage of reduction resulting from short-period exposure at 70° F. or 1 week at 50° was fully as great as with the unwaxed fruit. A similar reduction was obtained in the one test in which the waxed fruit was moved from 32° to 50° permanently at the end of 2 weeks.

In the experiments (table 8) in which browning of the oil glands, scald, or watery break-down occurred, the effect of the various moving treatments was far more pronounced than with pitting. It is interesting to note the number of cases in which there was a high percentage of scald or watery break-down on the controls and entire absence of the disease on the fruit that had been moved.

The data of tables 6 to 8, inclusive, were based on the condition of the fruit while it was still in storage, but the contrasts reported for the various diseases were fully maintained after removal from storage.

The fruit that had been heated was older in appearance and taste and this was occasionally true of fruit that had been delayed or that had been moved for short periods to higher temperatures.

The prestorage heating treatments often resulted in a decided increase in decay and the delays at room temperature sometimes resulted in a slight increase, whereas the various short-period removals to higher temperatures caused no increase in decay. The fruit that was moved to 50° F. permanently at the end of 1, 2, or 3 weeks' storage at the lower temperatures soon developed a very high percentage of decay; that moved at the end of 1 week showed much more decay than that moved at the end of 2 or 3 weeks.

The possible significance of the results from the various temperature changes is discussed later (p. 349).

Carbon Dioxide

A series of experiments was carried out to determine whether short-period exposure to carbon dioxide gas would have any effect upon the later development of pitting in low-temperature storage. The first tests were made on fruit described as lots 1A to 1F, inclusive (table 1). After shipment from Orlando, Fla., to Washington, D. C., by ordinary express (2 days) the fruit was given 2 days' exposure to atmospheres containing 40 to 45 percent of carbon dioxide gas before being placed in storage at 32° F. Similar lots were held at the same temperatures in normal air. Seven fruits were used under each condition. Most of the tests were made at 77°, 68°, and 49°, but two tests each at 50° and 41° were included. Individual records were kept of each fruit, as described on page 330. After 10 to 12

weeks in storage less definite pitting was found on the fruit receiving the prestorage carbon dioxide treatments in all of 18 tests and less mild pitting in 17 out of 18 tests. In both cases there was an average of one-third as much disease on the treated as on the untreated fruit. The contrasts were greater with the fruit that was held at the higher temperatures during the period of treatment than on that held at the lower temperatures.

In order to keep the percentage of carbon dioxide constant in the above experiments, the carbon dioxide-air mixture was continuously renewed, about five complete changes in the atmosphere being made in 24 hours, whereas the controls were held under moist-chamber conditions with no regulation of the air renewal, thus leaving the possibility of a difference in aeration as well as a difference in carbon dioxide. In later tests the fruit was held in pony refrigerators, with practically the same aeration conditions for the treated and untreated fruit but less accurate control of gas composition and temperature.

The pony-refrigerator experiments were made at Orlando, Fla., in the spring of 1932. Two crates of grapefruit were used under each condition. The refrigerators were cooled by ice, the temperature of the treated fruit dropping to 50° F. in about 18 hours and remaining there till the end of the experiment, and the temperature of the control fruit running 2° to 3° higher. The carbon dioxide was supplied from the usual type of carbon dioxide cylinder. It was run rapidly into the test refrigerator for 30 minutes and the rate was then decreased till it approximately balanced that of the leakage. In an experiment started on April 13 the carbon dioxide was held at about 20 percent and the treatment was continued for 30 hours. In an experiment started on April 16 the gas was held at an average of 35 percent and the treatment was continued for 20 hours.

The fruit was obtained direct from the grove, and after the treatment described it was shipped to the Arlington Experiment Farm, Rosslyn, Va., by ordinary express and was stored at 32° F. Notes were taken at various intervals on the development of disease. In the first experiment definite pitting on the control fruit ran about 50 percent higher than on the treated fruit; in the second experiment it ran about 90 percent higher. In both cases the treated fruit was more than 2 weeks behind the control fruit in developing a particular degree of definite pitting. In the first experiment there was little contrast between the treated and untreated fruit in the development of mild pitting, but in the second experiment it ran about 75 percent higher on the untreated fruit.

The reduction in pitting in the pony-refrigerator experiments was not as great as in the previous laboratory experiments, but the period of treatment was shorter and the percentage of gas lower. The 20-hour treatment with 35-percent carbon dioxide apparently gave better results than the 30-hour treatment with 20-percent carbon dioxide.

The flavor of the different lots of fruit was tested at various times and the treated fruit was found to be fully as good as the untreated fruit.

The results of the various carbon dioxide experiments seem to indicate that benefit can be obtained from the use of carbon dioxide as a prestorage treatment. Whether this benefit is due to a slowing down of metabolism, to the elimination of harmful products, or to some other cause has not been determined.

Paraffin and Cellophane Wrappers

In a few experiments fruit was held in paraffin or in moisture-proof cellophane wrappers in comparison with other fruit in standard paper wrappers (table 9). With cellophane wrappers definite pitting was reduced in 6 out of 7 tests and mild pitting in 5 out of 7 tests, the average of the 7 results showing a reduction of about 43 percent for definite pitting and 36 percent for mild pitting. With paraffin wrappers both forms of pitting were reduced in 6 out of 8 tests, the average of the 8 results showing a reduction of about 36 percent for definite pitting and 40 percent for mild pitting.

Friend and Bach (*5*) have reported a similar reduction of pitting with paraffin wrappers. Their fruit was apparently held at 32° F., whereas the lots just mentioned were held at 36° or 40°.

Oils and Waxes

In other experiments mineral oils and various combinations of oils and waxes were used in wrappers or on the fruit. The results are shown in tables 10 and 11.

Wrappers that were treated heavily with mineral oil decreased definite pitting in 14 out of 17 tests, the average of the results showing a reduction of about 52 percent. The application of mineral oil to the fruit reduced definite pitting in 14 out of 15 tests, the average of the results showing a reduction of about 65 percent.

The heavily oiled wrappers decreased mild pitting in 12 out of 17 tests, the average of the results showing a reduction of about 57 percent. The application of oil to the fruit decreased mild pitting in 12 out of 15 tests, the average of the results showing a reduction of about 70 percent.

The wrappers used in the foregoing experiments were more heavily oiled than could be used commercially, and the writers are of the opinion that some of the pitting reported in the wrapper tests was really oil injury. Commercially oiled wrappers, used in a few tests, gave only a slight reduction in pitting and caused no injury.

Table 9.—*Effect of paraffin and cellophane wrappers on pitting of grapefruit stored at low temperatures*

Lot no.	Fruits under each condition	Storage temperature	Storage humidity [1]	Storage period	Peel showing—					
					Definite pitting			Mild pitting		
					With paraffin wrappers	With cellophane wrappers	With untreated wrappers	With paraffin wrappers	With cellophane wrappers	With untreated wrappers
	Number	*°F.*		*Weeks*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
7	20	40	Low	8	------	2.40	6.1	------	1.20	2.90
8	8	36	High	6	0.35	.95	1.9	0.55	.42	.87
8	8	40	Low	10	.90	.90	1.2	2.50	3.20	6.10
9A	7	36	High	8	3.80	7.00	9.7	.74	1.50	1.70
9A	7	40	....do	8	3.50	------	5.1	.40	------	.17
9A	7	40	Low	8	3.30	4.70	3.8	.28	.65	.74
9B	7	36	High	8	5.90	3.40	4.0	1.80	1.70	.34
9B	7	40	....do	8	4.70	------	2.8	.65	------	1.00
9B	7	40	Low	8	4.30	5.40	13.2	.70	1.60	1.40

[1] "Low" indicates a relative humidity of 65 to 75 percent; "high", 85 to 90 percent.

TABLE 10.—*Effect of mineral oil on pitting of grapefruit*

Lot no.	Fruits under each condition	Storage period	Storage temperature	Peel showing—					
				Definite pitting			Mild pitting		
				With mineral oil		Control	With mineral oil		Control
				On fruit	In wrappers		On fruit	In wrappers	
	Number	*Weeks*	*°F.*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
2	26	9	32	----------	0.13	1.03	----------	0.03	0.92
3	21	9	32	----------	.17	.56	----------	.02	.81
6	8	10	36	2.30	2.10	3.60	0	0	.80
7	20	11	[1] 40	1.50	2.70	7.80	.95	2.00	2.70
8	8	10	36	0	0	.90	.78	.25	3.40
8	8	10	[1] 40	.90	.60	1.20	1.00	2.60	6.10
9B	16	8	32	.09	.27	.31	.30	.23	.16
9A	7	8	36	2.10	4.60	9.70	.90	2.70	1.70
9B	7	8	36	1.90	4.60	4.00	.74	0	.34
9A	7	8	40	1.70	4.10	5.10	.19	.40	.17
9B	7	8	40	2.00	6.00	2.80	.51	.40	1.00
9A	7	8	[1] 40	6.50	6.70	3.80	0	.28	.74
9B	7	8	[1] 40	4.80	5.40	13.20	.14	.25	1.40
10	8	7	40	.05	.75	2.60	0	.40	.08
10	8	7	[1] 40	.90	1.55	2.50	0	0	.28
11	8	7	[1] 40	----------	.42	2.20	----------	0	2.00
12	8	11	[1] 40	7.00	5.50	18.50	0	.40	.26
14	8	10	[1] 40	.60	----------	10.00	0	----------	.22

[1] With low humidity (65 to 75 percent).

TABLE 11.—*Effect of waxing on storage diseases of grapefruit*

Lot no.	Fruits under each condition	Storage period	Storage temperature	Peel showing—								Fruit showing watery breakdown	
				Definite pitting		Mild pitting		Browning of oil glands		Scald		Fruit waxed	Control
				Fruit waxed	Control	Fruit waxed	Control	Fruit waxed	Control	Fruit waxed	Control		
	Number	*Weeks*	*°F.*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
8	8	10	36	0	0.90	0.05	3.40	----------	----------	----------	----------	----------	----------
9A	7	8	[1] 40	.70	3.80	0	.74	----------	----------	----------	----------	----------	----------
9B	7	8	[1] 40	.77	13.20	0	1.40	----------	----------	----------	----------	----------	----------
10	16	7	40	.84	2.60	.18	.08	----------	----------	----------	----------	----------	----------
10	16	7	[1] 40	.47	2.50	.04	.28	----------	----------	----------	----------	----------	----------
11	8	7	[1] 40	.59	2.20	.15	2.00	----------	----------	----------	----------	----------	----------
12	8	11	[1] 40	4.30	18.50	.83	.26	----------	----------	----------	----------	----------	----------
13	8	13	[1] 40	.12	.50	.07	.12	----------	----------	----------	----------	----------	----------
14	8	10	[1] 40	.60	10.00	0	.22	----------	----------	----------	----------	----------	----------
15	9	9	36	.15	.78	0	.10	----------	----------	----------	----------	----------	----------
16	8	12	36	.05	3.60	0	.17	----------	----------	----------	----------	----------	----------
17	9	10	32	.05	.40	0	.13	0	0	2.5	2.30	0	0
17	9	10	36	.15	3.80	0	.30	0	0	0	0	0	0
18	24	12	32	0	.53	.30	.25	0	0	17.4	0	0	0
18	24	12	40	.05	.21	0	0	0	0	0	0	0	0
19	16	15	32	.04	.18	.67	.41	2.60	.70	0	0	0	0
19	16	15	36	.28	.71	.01	.10	.06	.02	0	0	0	0
20	24	13	32	.16	.30	.07	.30	11.80	4.50	0	0	0	0
20	24	13	36	.13	.50	.01	.13	0	.05	0	0	0	0
21	16	13	32	0	.22	.004	.05	0	0	0	0	43.8	12.5
21	16	13	36	1.00	4.40	.03	1.15	.48	.43	0	0	37.5	12.5
22	16	9	32	.15	.49	1.13	1.60	0	0	0	0	3.1	6.3
22	16	9	36	.13	.66	.12	.30	0	0	0	0	0	0
23	14	8	32	0	.02	.02	.01	0	0	12.1	.85	12.5	0
23	14	8	34	.06	.95	.004	.19	0	0	0	0	0	0
23	14	8	36	.02	.10	.14	.01	0	0	0	0	0	0
24	32	10	32	.02	.23	.05	.39	0	1.10	4.1	0	3.1	3.1
24	32	10	34	.17	.54	.05	.42	0	0	0	0	0	0
24	32	10	36	.05	.25	.05	.10	0	0	0	0	0	0
24	32	10	50	0	0	.004	.02	0	0	0	0	3.1	0
25	32	10	32	.02	.06	.48	1.80	0	0	5.2	1.30	21.9	3.1
25	32	10	34	.75	3.70	.50	.65	0	0	0	0	56.3	9.4
25	32	10	36	.71	3.10	.46	.72	0	0	0	0	21.9	9.4

[1] With low humidity (65 to 75 percent).

Fruit that was heavily waxed before storing developed less definite pitting than the controls in all of 32 cases where the disease was present in the controls, and the average reduction was about 82 percent (table 11). Heavy waxing resulted in the reduction of mild pitting in 26 out of a total of 33 tests, with an average reduction of about 63 percent. There was little contrast in the effect of waxing upon definite pitting at 32°, 34°, 36°, and 40° F. The reduction in mild pitting was greater at 36° than at 32° and 34°. The effect of the waxing upon both forms of pitting was more pronounced with low-humidity storage than with high-humidity storage.

The above contrasts in definite pitting were fully maintained when the fruit from 34°, 36°, or 40° F. was held for a week after removal from storage; i. e., more than five times as much definite pitting developed on the control fruit as on the waxed fruit in this period. In three-fourths of the lots stored at 32° the contrasts in definite pitting resulting from the waxing of the fruit were also maintained upon removal from storage, but there were a few definite exceptions.

The contrasts in mild pitting were fairly well maintained on the fruit from 36° and 40° F. upon removal from storage, but were largely lost on the fruit from 32° and 34°.

Browning of the oil glands, scald, and watery break-down were of sporadic occurrence, but there were several instances in which the last two of these diseases were very greatly increased as a result of waxing. Most of these instances were with fruit held at 32°. The modifying effect of temperature changes upon scald and break-down of waxed fruit is shown in table 8.

Experiments were made to determine the effects of delaying the application of wax for various periods after the fruit had been picked. Certain lots of fruit were waxed at Orlando at the time of packing, and comparable lots were similarly waxed 4 days later after shipment to Washington. In other experiments fruit was waxed after 1, 2, or 3 weeks' storage at 32° or 36° F. and compared with fruit that was waxed before storing at these temperatures. In all cases the results from delayed waxing were similar to those obtained from earlier applications.

The effect of waxing upon flavor was tested with various lots. There was seldom universal agreement among the persons making the tests, but the waxed fruit from 32° F. storage was usually rated lower than the control fruit from that temperature. The waxed fruit at 36° was often given as high rating as the control fruit and sometimes a higher rating. The fruit that had been heavily waxed seemed in some cases to be slightly less acid than the control fruit.

In most if not all of the above experiments the application of wax was heavier than would be practical under any commercial operation. In the later experiments an effort was made to secure a lighter waxing of the fruit. The lighter applications gave equally good results on the storage diseases, with little, if any, indication of any change in flavor.

The fruit that was waxed or wrapped in heavily oiled paper always looked fresher and had a better appearance than the control fruit. It also showed much less loss in weight. With high-humidity storage the control fruit usually showed a loss in weight of about 1 percent by the end of the first month, 2 percent by the end of the second month, and a much more rapid rate of loss with longer holding,

whereas the fruit that was waxed or wrapped in heavily oiled paper showed a rate of loss that was approximately 50 percent lower. With low-humidity storage the effect of waxing on loss of weight was much greater.

The waxes used in the above experiments consisted in all cases of a mixture of wax and oil combined in such proportion as to give a pliable product that could be readily rubbed on the fruit. Petrolatum and glycerin were occasionally included in the mixture, and water was sometimes added in an emulsion. Paraffin and carnauba were the usual waxes, and in some cases a small percentage of beeswax was added. Mineral oil was used in all instances. The writers have no best formula to recommend and are of the opinion that the modifying effects resulting from waxing are largely determined by the completeness of the covering.

In the experiments with lot 25, wax emulsions were tested in comparison with the wax mixtures reported above. The emulsions were prepared as follows:

A mixture consisting of 5 gallons of water, 0.9 pound of stearic acid, and 0.4 pound of triethanolamine was boiled gently with careful stirring until the acid was completely dissolved, giving a smooth soap solution. In a separate container 8.7 pounds of paraffin was melted over a hot water bath, care being taken that the temperature did not rise above 190° F. The melted paraffin was added to the boiling soap solution and stirred vigorously until an even dispersion of the wax was obtained; it was then stirred gently but continuously until cooled to room temperature.

Before being used, this emulsion was diluted, in some cases to 50, in some to 10, and in others to 1 percent of its original strength. The diluted emulsions were heated to 110° F. and the grapefruit was treated by rolling it around in the liquid for 1 to 3 minutes. In some cases the fruit was rubbed with a cloth after the emulsion treatment; in others it was dried before a fan.

In a few tests a modified emulsion was used to which beeswax and petrolatum had been added, and in another test ammonia was substituted for the triethanolamine.

The fruit that had received the various emulsion treatments was stored at 36° F. for comparison with similar untreated fruit and with fruit that had been coated with a mineral oil-wax mixture, as described above.

At the end of 10 weeks the waxed fruit showed a reduction in definite pitting of about 77 percent and a reduction in mild pitting of about 36 percent as compared with the control fruit, whereas the various emulsion treatments gave practically no reduction in either form of pitting. The waxed fruit showed a 30-percent reduction in loss of weight as compared with the control fruit, whereas the fruit that had received emulsion treatments showed an average of 6 percent reduction. There was no consistent variation in pitting nor in the loss in weight resulting from the different emulsion treatments.

EFFECT OF CHEMICAL VAPORS AND PEEL EXTRACTS

Grapefruit was found to respond to treatment with various chemical vapors. In the experiments reported in table 12 the fruit was held in 9-quart jars at room temperature during a 2-day treatment and later was stored at 32° or 36° F. The chemicals were dropped

into the jars from a pipette upon dry filter paper, and the jars were immediately closed.

The pitting produced by the vapor from the various chemicals was not always identical in appearance with that on the controls, yet the resemblance was close. It seems evident that many widely different chemicals are capable of producing pitting or of irritating the peel in such a manner that pitting follows.

In other experiments it was found that lemon oil applied in patterns to the peel of grapefruit occasionally produced injury in 32° and 36° F. storage. Steam-distillation extracts of grapefruit peel similarly applied produced injury resembling pitting in 8 out of 12 tests at both 32° and 40°. When grapefruit was held in closed jars at 70° and 3 to 6 cc of peel extract was introduced into the jars, slight but definite injury was evident in 4 days.

Puncturing the oil cells with a needle resulted in pitlike injuries, and in some instances there was a marginal browning that indicated injury from the oil released in puncturing.

Various attempts were made to produce similar effects by bruising the peel or by rubbing one grapefruit with the peel of another. In most cases the results were negative, but distinctly positive results were obtained with fruit from lot 25. The fruit had been in low-temperature storage several weeks before the experiment was started but had been warmed to room temperature before the treatments were given. Oil and juice were squeezed out of fresh grapefruit peel and rubbed on marked areas on other grapefruit, and the fruit was then stored at 31°, 32°, and 36° F. After 1 month's storage 18 out of a total of 48 treated areas showed injury, whereas the untreated areas remained free from injury. There was no significant contrast in the results at the different temperatures.

TABLE 12.—*Effect of various chemical vapors upon grapefruit*

Chemical	Quantity	Effect of treatment
	Cc	
Ammonia	0.8 to 1.7	Pitting greatly increased. Fruit more yellow in some cases.
Do	0.3 to 0.7	No contrast in pitting. Fruit more yellow in some cases.
Acetic acid	0.5 to 3.0	Pitting greatly increased; color decreased.
Do	0.3	No evident effect.
Alcohol, 95-percent	3 to 5	Do.
Acetaldehyde	0.4 to 0.5	Pitting greatly increased.
Do	0.3	No evident effect.
Formalin	0.3 to 1.7	Pitting greatly increased.
Petroleum ether	2.5 to 3	Pitting worse than in control.
Do	1 to 2	No evident effect.

The injury reported in table 12 was largely in the nature of definite pitting; that produced by lemon oil and grapefruit extracts was almost entirely of the oleocellosis type (fig. 8) but shading off in some instances into various degrees of pitting.

DISCUSSION

The foregoing experiments seem to give abundant proof that the seriousness of the storage diseases of grapefruit may be greatly modified by after-harvest treatment, yet the results have not always been consistent. Great care was taken in selecting and sampling the fruit

for treatment but it was found extremely difficult to make certain that the lots to be compared were physiologically similar. As grapefruit is seen in the picking crate or in the packing house there is considerable variation in the thickness of the peel and in the smoothness of its surface, and this physical variation is apparently indicative of differences in physiological response. The maturity of the fruit is also a disturbing factor from the standpoint of accurate experimentation. The grapefruit tree has a prolonged season of blooming, and fruits hanging side by side with no difference in appearance may be months apart in actual age. Fruit that is picked in the fall is likely to be from the early bloom and fairly uniform as to age, whereas fruit picked in the spring may be from the early, intermediate, or late bloom and represents a wide variation in age and probably in senility. From the standpoint of accurate experimentation the early pickings should be more desirable, but from the practical point of view it is the later pickings that should be studied because it is the late fruit that is likely to be stored.

With this background of variability it is not surprising that the results are not always consistent. It is not only possible that the individuals of a particular lot of fruit may have a wide variation in physiological condition but that the whole lot may be physiologically different from another lot, especially if grown in a different section or picked in a different season.

In spite of the variability in material and in results, certain principles regarding the storage behavior of grapefruit appear to have been established. Pitting has been decidedly increased by low humidity in the storage atmosphere, by the placing of soil or other foreign material inside the wrappers, and by the impregnation of the wrappers with glycerin. It has been decreased by high humidity in the storage atmosphere, by the use of cellophane, paraffin, or heavily oiled wrappers, and by coating the fruit with various oil-and-wax mixtures. All of these agencies have had a modifying influence upon the loss of moisture from the fruit, and it is the tentative opinion of the writers that this may be the main cause of their harmful or beneficial effects. This does not mean that pitting is to be considered a mere drying out of the peel. It is entirely distinct from shriveling and apparently is due to the effect that loss of moisture has upon enzyme or other physiological activities. A recent publication by Lauritzen and Balch (*10*) is significant in this connection. They found that the inversion of sucrose in the pile of sugarcane is intimately associated with the loss of moisture, increasing with the drying out of the cane. It seems probable in the present case that high humidity tends to delay some phase of destructive metabolism in the peel.

Nelson (*11*) has recently concluded that pitting of grapefruit is probably a manifestation of suboxidation. He was able to produce typical symptoms by holding the fruit in nitrogen at room temperature for 4 to 7 days. At temperatures of 31° to 33° and 38° to 42° F., pitting developed in 17 to 22 days when there was no deficiency of oxygen in the storage atmosphere. Two hypotheses were offered as possible explanations for the occurrence of the disease at low temperatures; one, that the oxidizing system is affected by low temperature to such an extent that there is a slow accumulation of toxic materials; the other, that low temperature may cause a preponderance of hydrolytic activity resulting in excessive splitting of substances like glucosides

and the consequent accumulation of materials that poison the protoplasm.

The data of the present paper give considerable support to Nelson's hypotheses and conclusions, yet there is much that can be interpreted otherwise.

Coating apples with oils and waxes or wrapping them in oiled paper is known to increase the carbon dioxide content and decrease the oxygen content of the internal atmosphere and the same thing is probably true for grapefruit, yet treatments of this type, as well as prestorage treatment with carbon dioxide, have resulted in a decrease in pitting. These facts do not seem to be in harmony with the theory of suboxidation yet they do not preclude the possibility that, other things being equal, a low oxygen supply may favor the development of the disease. Soft scald of apples has been classed as a suboxidation disease and, like pitting of grapefruit, is decreased by coating the fruit with wax or by prestorage exposure to high temperature or to carbon dioxide gas (*2*). This complete parallelism under such diverse treatments between diseases of two fruits so widely separated as the grapefruit and the apple makes a basis for interesting speculation, and would seem to form a background in the search for a fundamental cause, viz, some condition of metabolism that might be similarly modified by the various treatments.

Pitting of grapefruit and soft scald of apples are similar also in having definite temperature limitations, but their critical temperatures are different. Soft scald is most pronounced at a storage temperature of 30° to 32° F., and is largely eliminated by holding the fruit at 36° to 38°, whereas definite pitting is much worse at 36° than at 32°, but can be largely prevented by storing at a temperature of 45° to 50°. Whatever similarity there may be in the fundamental causes of the two diseases, it is evident that there are factors involved for which the temperature requirements are distinctly different.

Scald and watery break-down of grapefruit can be more readily classed as suboxidation diseases than pitting. They are increased by coating the fruit with wax and by subjecting it to high temperatures prior to storage, and their occurrence is especially favored by storage at 30° to 32° F.

Certain phases of the present report have important practical significance. Perhaps the most significant fact is the emphasis given to humidity. It has been commonly recognized that a high humidity is desirable in the storage rooms for grapefruit, but it is doubtful whether its extreme importance has been fully realized.

Rubbing a coat of wax (a mixture of wax and oil) on the fruit has greatly decreased pitting and decreased the loss in weight, but attempts to secure a satisfactory coating by means of wax emulsions have proved a failure under the conditions of the experiment (p. 346).

It has been found that grapefruit can be exposed to low temperatures for 2 weeks and sometimes for 3 or 4 weeks without the tendency toward physiological disorders becoming irreversibly established. Applications of wax at the end of 2 or 3 weeks' storage have been practically as effective in controlling pitting as have similar treatments before storage. Moving the fruit from 32° or 36° to 70° F. for 1 day or to 50° for 1 week at the end of 2 weeks' storage resulted in a great decrease in pitting, and moving it to 50° permanently at the end of 2 weeks resulted in practically complete elimination of pitting.

These results need further checking, but if it can be definitely established that grapefruit can be held for at least 2 weeks at a low temperature without developing a permanent tendency toward pitting it should make possible greater freedom in methods of shipment and storage, especially with fruit from sections where decay is a serious problem.

The knowledge that pitting, scald, and watery break-down are definitely decreased by removal to higher temperatures for short periods at the end of 1 or 2 weeks' storage at 32° or 36° might be of value at times in planning the transfer of fruit from shipping point to market-terminal storage.

SUMMARY

Descriptions are given for two forms of pitting and for scald, oleocellosis, browning of the oil glands, and watery break-down of grapefruit.

Bacteria were readily isolated from pitted tissue and certain forms were found capable of producing pits when inoculated into grapefruit peel but various modifying factors indicate that the disease is not primarily of bacterial origin.

Mild pitting was more pronounced and definite pitting about five times as bad at low as at high humidity. Both were increased by placing a small quantity of soil, sand, or charcoal inside the wrapper, or by impregnating the wrappers with glycerin, and all of these treatments resulted also in a decided increase in the loss of moisture.

Definite pitting was much more serious at 36° and 40° F. than at 32°, whereas mild pitting was somewhat worse at 32° than at 36° and 40°. Both were practically eliminated by holding the fruit at 50° F. Scald and watery break-down were worse at 30° and 32° than at 36° and 40°.

Holding grapefruit at temperatures of 60° to 75° F. before storing it at 36° resulted in a definite decrease in pitting, but holding the fruit at 50° before storing it at lower temperatures had no effect upon the later development of pitting. Prestorage heating for 17 to 22 hours at a temperature of about 100° resulted in a very great decrease in pitting in fruit stored at 36° or 40°, but in many cases gave a definite increase in fruit stored at 32°. Scald was much worse on heated fruit, especially that subsequently held at 32° storage.

Removing the fruit from room temperature for 1 day at the end of 1 week and again at the end of 2 weeks or moving the fruit to 50° F. for 1 week at the end of 2 weeks resulted in a decided reduction in both definite and mild pitting and also in scald and watery break-down. Removing the fruit to 50° permanently at the end of 1 or 2 weeks' storage at a lower temperature resulted in the practical elimination of physiological disorders.

Exposing grapefruit for 20 to 48 hours to atmospheres containing 20 to 45 percent of carbon dioxide before placing it in low-temperature storage resulted in a definite decrease in the later development of pitting, the best results being obtained with the longer treatments and the higher percentages of gas.

Storing the fruit in paraffin or cellophane wrappers gave a reduction in pitting similar to that obtained with initial carbon dioxide treatments.

Excessively oiled wrappers gave a much greater reduction in pitting than paraffin or cellophane wrappers.

Coating the fruit with mixtures of mineral oil and wax resulted in a much better control of pitting than any type of wrapper used but sometimes caused an increase in scald and watery break-down, especially with heavy coating and 32° F. storage.

Fruit held at 50° F. soon developed a very high percentage of stem-end rot, whereas fruit held at 40° or lower usually showed no decay at the end of 8 weeks' storage.

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EXTENT OF VERTICAL MIGRATION OF HORSE STRONGYLE LARVAE IN SOILS OF DIFFERENT TYPES [1]

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INTRODUCTION

Plowing under of soil infested with eggs and larvae of livestock parasites is generally considered a useful prophylactic measure against worm parasites. However, in 1932, Hall (*2*) [2] called attention to the lack of adequate information concerning the ability of nematode larvae to migrate vertically in soil, and emphasized the need for experimental investigations on this subject. The experiments described in this paper were undertaken to obtain information on the extent to which the vertical migration of horse strongyle larvae in soil occurs. Such information is closely related to the value of plowing under as a control measure for horse strongyles.

REVIEW OF LITERATURE

Since Ransom's (*5*) investigation on the life history of *Haemonchus contortus*, published in 1906, various investigators have noted a tendency on the part of infective strongyle larvae to climb up the walls of glass containers when a film of moisture was present on the sides of the culture vessels. Nöller and Schmid (*4*) reported that horse strongyle larvae commonly show this tendency. Enigk (*1*) stated that these larvae climb up stable walls which have been treated with milk of lime, provided the boards are moist. Wetzel's comment on Hüber's paper (*3, p. 710*) noted that horse strongyle larvae climb up grass blades in wet or damp weather and return to feces or soil as sunshine dries the grass. Schwartz, Imes, and Wright (*7*) make the following statement regarding the vertical migration, on grass, of larvae of the large horse strongyles:

> When the air is sufficiently moist so that the grass becomes covered with a film of moisture, as happens in times of rain, dew, or fog, the larvae migrate up the grass blades and this brings them to a favorable situation to be swallowed by horses while grazing.

This statement was based on observations by Schwartz [3] who, by means of the Baermann apparatus, isolated horse strongyle larvae from those portions of blades of grass which had no contact with the soil, the larvae being especially abundant on the grass after dews or rains. Similar observations have been reported in connection with the behavior of larvae of other strongylid nematodes.

So far as is indicated by examination of the literature dealing with the behavior of horse strongyle larvae, the vertical migration of these larvae in soil has not been previously investigated. In fact, investi-

[1] Received for publication Oct. 2, 1935; issued April 1936.
[2] Reference is made by number (italic) to Literature Cited, p. 361.
[3] Personal communication to the author.

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gations concerning the vertical migration in soil of the larvae of strongyles of domestic animals have been confined to only a few species. Ross and Kauzal (*6*) recently reported experiments which indicate that the infective larvae of *Stephanurus dentatus*, a species parasitic in swine but reported once from an equine, showed very little tendency to reach the surface of mud when buried to depths varying from about 5 to 7.5 cm; Spindler (*9*) buried several hundred infective larvae of *S. dentatus* beneath 2.5 inches of moist soil in each of 12 tin cans; only 9 larvae were recovered from the surface soil in the cans during the course of daily examinations over a period of 3 weeks. Spindler did not find larvae of this parasite in six weekly examinations of the surface soil from a freshly plowed pasture which had harbored numerous *Stephanurus* larvae before the land was plowed. The same investigator (*8*) obtained a similar result in an extensive field investigation involving the vertical migration in soil of the larvae of *Oesophagostomum* of swine.

METHODS USED IN THIS INVESTIGATION

Freshly passed horse feces, uncontaminated with bedding or soil, were collected and cultured in glass containers at room temperature (69° to 76° F.). Infective larvae were obtained from these feces by means of the Baermann isolation apparatus.

Soils varying as much as possible in type were obtained near Beltsville, Md. Screened soil of the desired type was rendered helminthologically sterile by heating it in open pans until all of it reached a temperature of at least 156° F. In some tests unheated soil was used; this soil was taken from localities to which, so far as is known, horses had had no access. Moreover, before unsterilized soil was used in the tests, samples were placed in the Baermann apparatus and no horse strongyle larvae were found in them.

The sterilized and tested unsterilized soils were moistened with water and firmly packed into containers, usually wooden boxes. The boxes either had wooden bottoms in which a number of holes were bored, or bottoms of fine wire screen in order that water might pass downward through the soil in a normal manner. Excavations about 1.5 inches in diameter and of the desired depths were made in the center of the soil in the containers.

Except in one experiment, no. 8, larvae which had attained the infective stage not more than 1 or 2 weeks previous to the commencement of the experiment, suspended in a few cubic centimeters of water, were placed at the bottom of the excavations by means of a long glass pipette. A few minutes were allowed to permit absorption of the water by the soil before the excavations were refilled with the previously removed soil, which was carefully compressed. In all the experiments except no. 8, the larvae placed in the soil were counted, a dilution method being employed for this purpose.

In making the vertical-migration studies, 10 experiments were carried out. Experiments 1 and 3 were performed in the laboratory. Glass tubes about 8 inches high and 6 inches in diameter, open at both ends and resting on tin plates, were used as containers for the soil. After the larvae had been buried, lids were placed over the mouths of the tubes and the preparations were placed in a dark cupboard where they remained throughout the duration of the experiment.

Experiments 2, 5, 7, 8, and 9 were made outdoors on small fenced plots in the vicinity of Beltsville, Md. In experiments 2, 7, and 9, wooden boxes about 13 inches square and 8 inches deep were used as containers for the soil. The boxes were sunk in sandy soil in such a manner that their upper edges protruded 1 inch above the surface of the ground, and the level of the soil in the boxes was about the same as that of the surrounding soil. In experiment 5, boxes about 17 inches square, having a depth of either 4 or 8 inches, were used. They were placed in shallow excavations made in sandy soil. In experiment 8, four bottomless wooden frames, about 15 inches square and 10 inches deep, were sunk in sandy soil to such a depth that their upper edges protruded about 1 inch above the surface. The soil was completely removed from within the frames and replaced with tested sand. Pellets of horse feces containing large numbers of infective horse strongyle larvae were buried at the bottom of excavations, about 3 inches in diameter, prepared in the soil within each frame.

Experiments 4, 6, and 10 were carried on inside a small wooden shelter located on a shaded lot. The shelter afforded complete protection from rain, and the temperature within the shelter varied in accordance with the temperature of the outside air. In each experiment, two wooden boxes about 13 inches square, one of which was 4 inches and the other 8 inches deep, were used as containers for the soil. Tall metal cylinders about 12 inches in diameter were pressed into the soil to a depth of about one-half inch to prevent larvae from migrating out of the boxes as a result of possible lateral movement in the surface layer. A large metal box, about 6 feet long, 4 feet wide, and 15 inches deep, was filled with moist soil, and the wooden boxes were sunk in this soil in such a manner that the level of the soil within and without the wooden boxes was approximately the same. Water not only was sprinkled on the surface of the soil in the wooden boxes from time to time but also was poured into a short metal pipe located in one corner of each box.

In all experiments in the laboratory or under shelter, the soil was kept moist by the addition of water from time to time. The variation in the temperature of the laboratory has been indicated previously. In the outdoor experiments in which no shelter was provided, water was not added to the soil in the boxes after the larvae were buried. Precipitation invariably occurred during the period in which the experiments were in progress.

At the expiration of the desired interval, the entire surface layer of the soil in the container was scraped off with a spoon or spatula to a depth of about one-eighth of an inch to collect larvae that had migrated to the surface. The scrapings were placed in the Baermann apparatus, and after 24 hours or longer the fluid was withdrawn from the bottom of the rubber tubing and the larvae recovered were counted.

EXPERIMENTAL DATA

VERTICAL MIGRATION AS AFFECTED BY TYPE OF SOIL

The data pertaining to the vertical-migration experiments with different types of soil (table 1), show that in from 19 to 53 days there was extremely little migration of horse strongyle larvae to the surface of clay soil from depths of 1 to 6 inches. In heavy sandy loam to clay

TABLE 1.—*Results of experiments on vertical migration of horse strongyle larvae in types of soil indicated* [1]

Soil type	Experiment no.	Location of experiment	Container no.	Approximate number of larvae buried	Depth of burial	Date of burial	Time from burial of larvae to examination of surface soil	Larvae recovered from surface soil		Meteorological data [2]	
										Mean temperature	Total precipitation
					Inches		*Days*	*Number*	*Percent*	°*F.*	*Inches*
HEAVY SOIL											
Clay	1	Indoor	1	28,000	1	Jan. 25, 1933	19	7	0.02		
			2	28,000	2	....do....	19	0	0		
			3	28,000	2	....do....	53	0	0		
	2	Outdoor	1	[3]	2	May 16, 1934	23	0	0	69	3.09
			2	45,000	3	....do....	23	2	.004	69	3.09
			3	45,000	6	....do....	23	0	0	69	3.09
MODERATELY HEAVY SOIL											
Heavy sandy loam to clay loam	3	Indoor	1	50,000	1	Nov. 16, 1932	26	10,500	21.0		
			2	38,000	2	Nov. 15, 1932	27	1,500	3.9		
			3	57,000	4	....do....	28	2	.003		
			4	57,000	5	....do....	28	0	0		
Sandy clay loam	4	Outdoor, under shelter	1	40,000	2.5	July 25, 1934	44	1,427	3.5	74.3	
			2	40,000	5.5	....do....	37	69	.17	75	
MODERATELY LIGHT SOIL											
Sandy loam	5	Outdoor	1	12,000	1	Jan. 30, 1933	63	25	.2	41	6.09
			2	20,000	2	Jan. 26, 1933	67	75	.37	41	6.87
			3	70,000	3	Feb. 6, 1933	60	250	.36	42	6.62
			4	16,000	5	Feb. 21, 1933	62	12	.07	47	8.13
			5	93,500	6	July 27, 1933	41	85	.09	76	10.90
Do	6	Outdoor, under shelter	1	40,000	2.5	July 25, 1934	44	407	1.0	74.3	
			2	40,000	5.5	....do....	37	11	.027	75	
Sandy light clay loam	7	Outdoor	1	45,000	2	May 8, 1934	23	857	1.9	67	2.72
			2	45,000	3	May 3, 1934	25	848	1.9	67	3.54
			3	45,000	6	....do....	25	550	1.2	67	3.54

LIGHT SOIL

Coarse sand	8	Outdoor	1	(4)	[5] 3	Sept. 29, 1933	27	7,190		58.6	2.87
			2	(4)	[5] 5	do	27	12,704		58.6	2.87
			3	(4)	[5] 6	do	28	9,045		58	2.87
			4	(4)	[5] 8	do	28	6,920		58	2.87
	9	do	1	45,000	2	May 3, 1934	28	22	.05	69	3.67
			2	45,000	3	do	28	232	.51	69	3.67
			3	45,000	6	do	28	53	.12	69	3.67
	10	Outdoor, under shelter	1	40,000	2.5	July 25, 1934	44	277	.69	74.3	
			2	40,000	5.5	do	37	42	.10	75	

[1] Soil designations by J. E. Lapham of the Bureau of Chemistry and Soils, U. S. Department of Agriculture.
[2] Taken from reports of the Washington, D. C., station of the Weather Bureau, U. S. Department of Agriculture, located approximately 13 miles southwest of Beltsville, Md.
[3] Number undetermined but exceeded 45,000.
[4] Fecal pellets containing larvae were buried. It was impractical to determine the number of larvae present.
[5] The depth given is that of the column of sand above the uppermost surface of the fecal pellets.

loam and sandy clay loam, very few of the larvae buried to depths of 4 to 5.5 inches reached the surface in intervals ranging from 28 to 37 days; from depths of 1 to 2.5 inches, however, appreciable numbers of larvae reached the surface in from 26 to 44 days. In sandy loam and sandy light clay loam, small percentages reached the surface from depths of 1 to 6 inches in from 23 to 67 days. Likewise, small percentages reached the surface of coarse sand from depths of 2 to 6 inches in from 28 to 44 days. The results of experiment 8, although the larvae buried were uncounted, indicate that considerable numbers reached the surface of coarse sand in 1 month when small quantities of horse feces were buried from 3 to 8 inches beneath the surface. In general the individual experiments show that the percentage of larvae which reached the surface varied inversely with the depth of burial.

VERTICAL MIGRATION AS AFFECTED BY LATERAL SUBSURFACE MIGRATION

Since the soil containers used in the vertical-migration experiments were very limited in size, it was desirable to know whether the results might have been affected by lateral migration of the larvae beneath the soil surface to the edges of the containers, followed by ascent along the surface of the containers. Two experiments were performed to determine the extent of such migration, sandy light clay loam being used in each experiment.

In the first of these experiments, a covered wooden box 7¼ inches long, 6 inches wide, and 6½ inches deep, was filled with soil. The box was so constructed that sections of the sides could be withdrawn without disturbing the position of the moist soil. On March 24, 88,000 larvae were buried to a depth of 4 inches in the center of the soil. The distance from the sides of the rectangular excavation, in which the larvae were placed, to the sides of the box was 2¼ inches. On April 10, 17 days after the experiment began, 66,500 larvae were recovered from the surface soil. Of these larvae, 143 were in the soil directly above the area where the larvae were originally buried, 904 were in the surface soil within a distance of about 1 inch from the central rectangular area, and the remainder were recovered from the area extending from this point to the sides and ends of the box. Sections of one side of the box were removed, and 42 larvae were found in the soil of the lateral surface at an average distance of 3½ inches from the top; 16 larvae were found at an average distance of 1¼ inches from the top. On the opposite side, 25 larvae were found in the lateral surface soil at a distance of about 2 inches from the top. Larvae were isolated also from the surface of the wooden sections removed from the box.

In the second experiment a wooden box about 17 inches square and 6 inches deep, resting on a thin layer of moist soil in a large metal tray, was filled with soil. About 94,000 larvae were buried in a central excavation 1 inch in diameter and 2½ inches deep. A metal cylinder 2½ inches in diameter was placed directly over the spot beneath which the larvae were buried; the lower edge of the cylinder was pressed into the soil to a depth of about one-half inch. In a similar manner, tall metal cylinders, 5, 8, and 10 inches in diameter, were placed concentrically about the small cylinder. Barriers were thus erected to prevent the possibility of migration of larvae laterally on the surface of the soil, the arrangement permitting a determination and comparison

of the number of larvae reaching the surface in each of the five separate areas so produced. After 19 days the surface soil from the area extending from the edges of the largest cylinder to the sides of the box was removed, and after 21 days the surface soil from each of the remaining areas was removed, each sample being separately examined. The results are shown in table 2.

TABLE 2.—*Results of experiment to determine effect of lateral subsurface migration on vertical migration of horse strongyle larvae*

Area no.	Larvae recovered [1]—		Area no.	Larvae recovered [1]—	
	From surface soil	Per square inch of surface		From surface soil	Per square inch of surface
	Number	*Number*		*Number*	*Number*
1 (center)	3,463	707	4	2	0.07
2	3,043	206	5 (outer)	8	.04
3	18	.58			

[1] The total number of larvae buried was 94,000.

The data from the two experiments just described indicate that lateral migration beneath the soil surface occurs during the ascent of the larvae to the surface layer. In reaching the surface from a depth of 2½ inches, in the second experiment, a few larvae migrated laterally 4½ inches or more, but the vast majority did not migrate more than 2 inches laterally during their ascent. In this experiment approximately 7 percent of the buried larvae reached the surface from a depth of 2½ inches in from 19 to 21 days. In the first experiment, about 75 percent of the buried larvae reached the surface in 17 days.

DISCUSSION

It is difficult to compare the writer's results with those of Ross and Kauzal (*6*) and Spindler (*9*) in their experiments with larvae of *Stephanurus dentatus*, since these investigators did not definitely mention the type of soil used. Spindler (*8, 9*) concluded from his field studies that plowing the soil under and later growing a crop on it frees the land of *Stephanurus* and *Oesophagostomum* larvae; this procedure is regarded by Spindler as an effective control measure for these parasites.

In the writer's experiments, horse strongyle larvae migrated vertically in coarse sand, sandy loam, sandy light clay loam, sandy clay loam, and heavy sandy loam to clay loam, distances equivalent to those attained by shallow to average plowing (from 4 to 6 inches). However, from such depths only from 0.003 to 1.2 percent of the buried larvae reached the surface of these soils in from about 3 weeks to 2 months. If it is assumed that the rates of migration indicated by the experiments for the periods mentioned remain approximately constant, it is evident that only a small percentage of the larvae actually buried 4 inches or deeper would reach the surface of the soil in the course of a year. However, the writer's experiments showed also that a considerable percentage of buried larvae quickly reached the surface from shallow depths, particularly in loam soils. It is evident, therefore, that although plowing under of infested soil

undoubtedly possesses considerable value as a control measure for strongylidosis in the horse, the efficacy of this control measure has definite limitations, being affected by the depth to which the larvae are buried and the type of soil in which they are present. It is indicated by the experiments that if larvae are buried in clay soil by plowing, very few will subsequently reach the surface.

The experiment described on page 358, in which a very large percentage of buried larvae quickly reached the surface of sandy light clay loam soil from a depth of 4 inches, may appear to present a result inconsistent with certain of the above statements. The succeeding experiment (p. 358) suggests, however, that the larvae migrated laterally beneath the surface of the soil to the sides of the box and ascended these moist surfaces to the upper soil layer. Enigk (*1*), as already stated, has shown that horse strongyle larvae migrate vertically along moist wooden surfaces. Such upward migration probably occurs on plant roots. The results of these two experiments indicate that lateral subsurface migration was not a factor seriously influencing the results of the writer's experiments on vertical migration.

SUMMARY AND CONCLUSIONS

Experiments performed indoors and outdoors indicate that there is practically no vertical migration of horse strongyle larvae in clay soil.

Experiments made indoors and outdoors under shelter showed that extremely small percentages of buried larvae reached the surface of heavy sandy loam and sandy clay loam from depths of 4 to 5.5 inches in from about 4 to 6 weeks. During similar periods, 3.5 percent or more of buried larvae reached the surface of these soils from depths of 1, 2, and 2.5 inches.

Both indoor and outdoor experiments involving sandy loam and sandy light clay loam soils showed that from 0.027 to 7 percent of buried larvae reached the surface from depths of 1 to 6 inches in from 19 to 67 days; in general, the percentage of larvae reaching the surface varied inversely with the depth to which they were buried. An experiment in which about 75 percent of the buried larvae reached the surface in less than 3 weeks is excepted in these statements because the data indicate that the larvae ascended the moist wooden sides of the small box in which the soil was kept. Larvae probably could ascend plant roots in the same way.

Small percentages of larvae reached the surface of coarse sand from depths of from 2 to 6 inches in from 28 to 44 days. In a nonquantitative experiment involving coarse sand, appreciable numbers of larvae reached the surface from depths as great as 8 inches in about 4 weeks.

Experiments involving sandy light clay loam showed that during vertical ascent to the surface some larvae migrated laterally for short distances beneath the soil surface.

Deep plowing under of infested soil should be of some value for the control of strongyle parasitism in horses, but the efficacy of this control measure is subject to definite limitations, particularly in respect to the depth at which the larvae are buried by plowing and the type of soil in which they are present. Clay soil presents an almost complete barrier to the vertical movement of the larvae.

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ZINC AND OTHER MINERAL CONSTITUENTS IN RELATION TO THE ROSETTE DISEASE OF PECAN TREES[1]

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INTRODUCTION AND REVIEW OF LITERATURE

Rosette is a physiological disease of pecan trees. Symptoms are variable but usually include a mottled chlorosis and crinkling of newer tipmost leaves and shortening of the internodes of new shoot growth. Affected leaflets do not attain normal size and are frequently malformed. A necrosis and abscission of leaflets and entire leaves may follow. The resulting leafless shoots frequently die back. New growth arises below the dead area. This usually becomes affected in the same or subsequent growing season. The tree becomes misshapen and worthless, though it seldom dies outright. The disease occurs widely throughout the Southern States. Its presence was reported in Arizona in 1911.

In studies of pecan rosette in Arizona previously reported (*8*),[3] it was found that both strongly and weakly vegetative trees are affected. This fact was interpreted as meaning that factors responsible for rosette are somewhat independent of those governing length of vegetative extension. Amount and length of growth in plants have been reported by many workers to be closely related to carbohydrate and nitrogen nutrition in the plant. If imperfections in nutrition were responsible for rosette, they would be of a mineral rather than of an organic origin. Some ions and elements are not readily available in alkaline soils. Occurrence of rosette is common on, but not limited to, alkaline soils.

Similar chloroses and malformations in other plants have been induced by growing the plants in a nutrient deficient in certain ions (*4*, *11*) or, where occurring in the field, have been corrected by application of minerals not supplied in ordinary fertilizers (*2*, *10*, *21*).

As organic nutrition is unlikely as a cause, and some ions may not be satisfactorily available in the alkaline soils of Arizona, and also since rosetted trees display symptoms similar to those in other plants which were corrected by the addition of certain ions, the conclusion was that rosette might be due to a mineral deficiency. To test for a mineral deficiency, the most effective procedure is to use many differing elements and ions and to apply them singly, not to the soil where fixation might render them unavailable to the tree or where a response of the tree to them might be slow or indefinite, but rather to apply them directly to the aboveground parts of the tree where their effect might be readily noted. This procedure revealed that pecan rosette yielded to treatment with zinc (*8*). A

[1] Received for publication July 9, 1935; issued April, 1936.

[2] The writer is indebted to the members of the Department of Agricultural Chemistry, University of Arizona, who kindly permitted the use of their laboratory and equipment for conducting the analyses described herein. Especially is gratitude extended to Dr. R. A. Greene, who gave counsel throughout the analytical procedure.

[3] Reference is made by number (italic) to Literature Cited, p. 375.

similar method was used by Alben et al. (*1*). Other workers have reported a curative action of zinc (*6*).

The various data leave little doubt but that pecan rosette yields to treatment with zinc. Before this can be finally concluded, a strictly chemically pure form of zinc must be used. As far as the writer is aware no worker has yet used such a material. The fact that similar symptoms in peach, plum, apricot, grape, walnut, and orange in California (*5*), cherry in Oregon (*15*), citrus in Arizona (*7*), and tung, citrus, and corn in Florida (*17*) respond similarly to zinc indicate a common causal relation of the various diseases.

Rosetted pecan trees in Arizona respond readily to the application of zinc sulphate to the soil around the trees (*9*), and are now being treated satisfactorily in that way. In the alkaline soils of Arizona, pecan trees are not injured to any appreciable extent by a toxic action of zinc sulphate in the soil. Similar treatment of citrus mottleleaf is not practical here because of the toxic action of zinc on the trees, as has been reported by Parker (*18*) in California. Further information on the occurrence and availability of zinc in the soil may lead to the devising of cultural methods to make zinc more available and thereby effect a remedy without the actual addition of zinc. The present paper reports experiments in which zinc was used in treating pecan trees affected with rosette.

THE PROBLEM

With the recognition that pecan rosette is in some way associated with zinc, interest focuses upon the occurrence and action of zinc in the plant, particularly in view of certain commonly observed phenomena. The top of the tree is most susceptible to rosette. Is this because of less zinc in the tissue? Similarly, why may one limb be healthy and another on the same tree be affected? Why may one tree be affected in an otherwise healthy orchard, and vice versa? Why do trees healthy for many years suddenly become affected or trees diseased for many years become healthy with or without change in cultural treatment? Is this because of a change in the total amount of zinc in the tissue or is it a question of availability of zinc for physiological use? Is the availability of zinc modified by the presence of other elements or ions in the tissue? To gain initial information upon the occurrence, distribution, movement and availability of zinc, analyses were made of pecan tissue for ash, silica, calcium, magnesium, iron, copper, and zinc in 1933, and for ash and zinc in 1934.

EXPERIMENTAL METHODS AND MATERIALS

SEASON OF 1933

An 8-year-old orchard of Burkett pecan trees growing near Tucson, where rosette has been generally severe, was used. One previous sampling of the irrigation water revealed an absence of zinc (*8*). The soil had a pH value of approximately 8 and contained about 200 p. p. m. of total soluble salt. Most of the trees in the orchard were small and misshapen from rosette, but several, including those sampled, were of ordinary size for their age. All displayed evidence of having had rosette in some previous years. The healthy untreated tree used for sampling was apparently not far removed from a rosetted

condition, for while it showed no symptoms at time of sampling, it displayed mild chlorosis by late August. This may mean that if the present studies measured factors related to the cause of rosette, the widest possible extremes between healthy and affected tissue were not represented in these samples.

On June 22 four trees were selected. Two of these had chlorotic leaves in the topmost parts, but there was no necrosis of shoots. The other two trees showed no symptoms of rosette. One diseased and

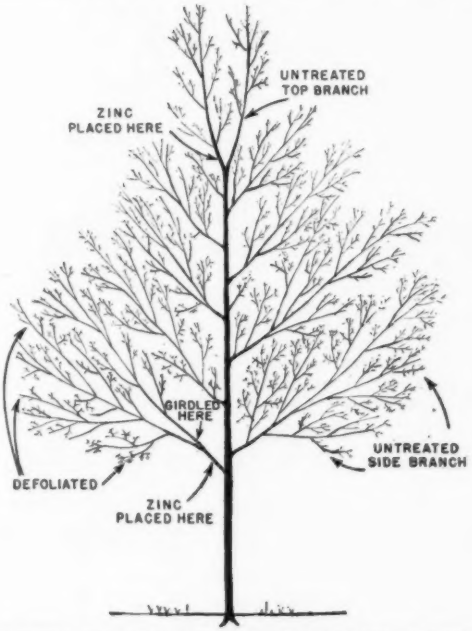

FIGURE 1.—Diagram of tree given special treatment as described in the text. The applications of zinc, the defoliation, and the girdling were done on July 14. Samples for analysis were collected on August 18. The defoliation was not repeated so that new growth had its full complement of leaves when sampled.

one healthy tree was treated by placing approximately 30 g of commercial zinc sulphate ($ZnSO_4.5H_2O$) in three holes bored into the trunk.

On July 11, samples were collected from each of the four trees. Samples consisted of (1) the top 6 inches of 1933 season's growth of topmost branches and leaves, (2) a similar portion of lower outside branches, and (3) entire 1933 season's growth of short inside shoots. The two trees healthy on June 22 were still so when sampled. Of the affected trees on that date, the treated one was making healthy growth; whereas leaves of the top branches in the untreated one were severely chlorotic and there was some necrosis of leaves and shoots. The samples collected of outer side branches were mildly chlorotic. The short inside shoots were healthy.

On July 14 a fifth mildly affected tree having a height of only 15 feet was treated as shown in figure 1, 10 g of zinc sulphate being used

for each application. On August 18 samples for analyses were collected from this tree as follows:

(1) New growth on girdled branch. Shoots here had made only 2 inches of growth since girdling. There were no symptoms of rosette and no union of cambial or cortical tissue at the girdle.

(2) New growth on defoliated branch. The defoliating had been done only once so that new growth had its full complement of leaves. Shoots had made 6 inches or more of growth since defoliation. No symptoms of rosette.

(3) New growth on an untreated side branch, check for 1 and 2. Samples displayed mild chlorosis of leaves, but shoots were not necrotic.

(4) New growth on treated branch in top of tree. No symptoms of rosette.

(5) New growth on untreated branch in top of tree. Leaves were mildly chlorotic, but no necrosis of leaves or shoots were found.

SEASON OF 1934

On October 16, 1934, samples were collected of what was thought might be wider differences of healthy and affected tissue than were represented in the samples of 1933. These consisted of leaves collected at the tips of 1934 season's growth in the top of 10 rosetted and 9 healthy trees in the Yuma Valley. The rosetted trees were 5 to 8 years of age and located in four orchards along the east side of the valley. Rosette has been common there. The trees sampled were known to have been rosetted for the preceding three summers. The healthy trees were from a single 8-year-old orchard on the west side of the valley where rosette has been unknown.

CHEMICAL ANALYSES

All samples were washed in distilled water as soon as collected and were subsequently dried in the sun or in the draft of an electric fan and stored in the laboratory until analyzed during the winter following collection. The samples were broken into small pieces, placed in a silica or platinum dish, dried overnight at 110° C., and weighed. This was the dry weight upon which all percentages were based. Leaf samples generally weighed from 90 to 100 g, and stem samples 20 to 40 g. Samples were ashed at dull redness in an electric muffle. The ash was weighed, then taken up with dilute hydrochloric acid. Analyses were made upon aliquots of this for the various elements using the methods of the Association of Official Agricultural Chemists (*3*).

This method for the determination of zinc consists of separating zinc from copper by precipitation of the copper with hydrogen sulphide in solution, containing 5 cc of concentrated hydrochloric acid in 250 cc, then by precipitating zinc as the sulphide with the solution adjusted to a pH of 2 to 3. The zinc is estimated turbidimetrically as the ferrocyanide. It is recognized that the method is not completely accurate. The micromethod described by Hibbard (*13*) is probably an improvement, and he provided a copy of his method before analyses were begun on the material collected in 1934. It was deemed best to continue with the official method since it had been used the two previous years. Furthermore, the use of large samples (18 to 20 times as large as those suggested by Hibbard) would tend to reduce errors incidental to analyses.

DISCUSSION OF DATA

The results as found for healthy and rosetted pecan trees, untreated and treated by placing zinc sulphate in the trunk are shown in table 1 and figure 2. Similar data for some parts of the tree given special

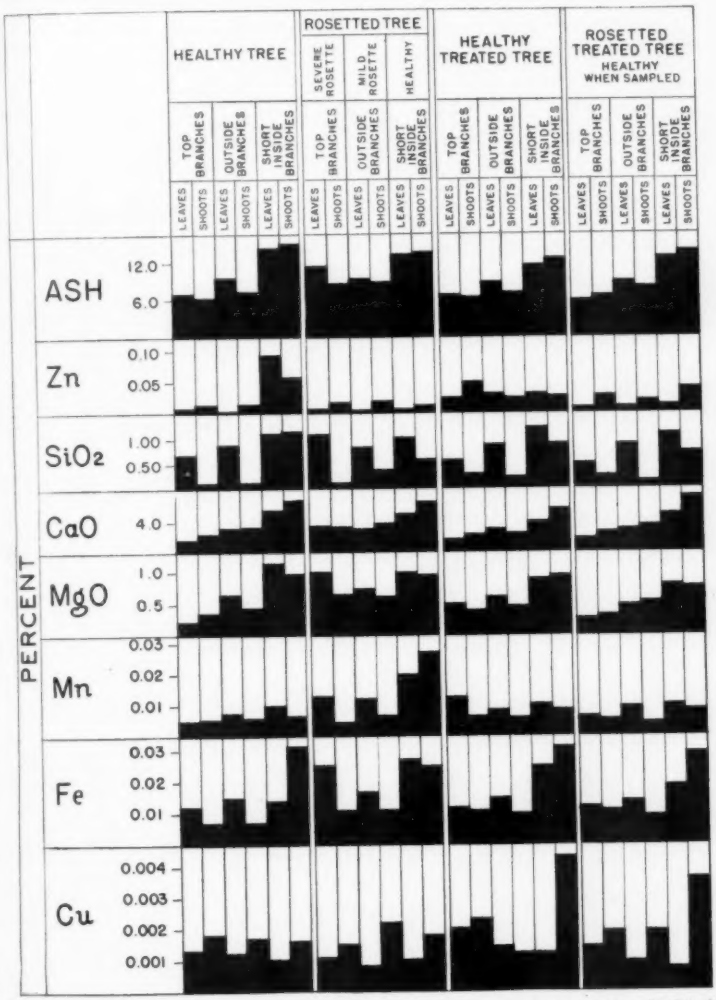

FIGURE 2.—Analytical data from several trees sampled in 1933. These trees were growing in a district where rosette has been common. The healthy, untreated tree was probably close to a rosetted condition when sampled, for mild symptoms appeared later in the summer.

treatments as shown in figure 1 are shown in table 2 and figure 3. The data for zinc and ash content of the healthy and diseased tissue collected in the Yuma Valley in 1934 are shown in table 3 and figure 4.

The zinc content was also calculated as the percentage of total ash in the tissue; this tended only to show wider differences in zinc content and the data computed in this way are not included.

TABLE 1.—*Percentage of oven-dry weight, ash, and certain mineral constituents in tissues of healthy and rosetted trees when sampled July 11, 1933*

Condition of tree	Branches sampled	Condition	Tissue	Ash	Silica	CaO	MgO	Mn	Fe	Cu	Zn
Healthy	Top	Healthy	Leaves	7.12	0.553	1.83	0.2306	0.0056	0.0129	0.0013	0.0088
			Shoots	6.42	.108	2.29	.3811	.0058	.0075	.0018	.0143
	Side	do	Leaves	9.72	.724	3.09	.6612	.0078	.0154	.0012	.0066
			Shoots	7.92	.114	3.13	.4476	.0062	.0077	.0017	.0149
	Short inside	do	Leaves	14.46	.916	5.45	1.1450	.0102	.0140	.0010	.0932
			Shoots	15.12	.930	6.77	.9906	.0067	.0319	.0016	.0575
Rosetted [1]	Top	Severe rosette	Leaves	11.54	.886	3.51	1.0192	.0134	.0253	.0011	.0076
			Shoots	8.45	.105	3.49	.6644	.0048	.0110	.0015	.0187
	Side	Mild rosette	Leaves	9.29	.680	3.03	.7552	.0123	.0171	.0008	.0062
			Shoots	8.49	.319	3.73	.6367	.0061	.0113	.0022	.0191
	Short inside	Healthy	Leaves	13.16	.837	4.95	1.0726	.0200	.0270	.0010	.0074
			Shoots	13.76	.475	6.39	.9763	.0272	.0250	.0018	.0125
Healthy, treated [2]	Top	do	Leaves	6.50	.469	1.69	.5177	.0129	.0120	.0020	.0251
			Shoots	6.32	.245	2.19	.4174	.0063	.0112	.0023	.0500
	Side	do	Leaves	8.47	.717	2.91	.6223	.0084	.0158	.0014	.0318
			Shoots	6.88	.192	2.31	.4622	.0061	.0103	.0012	.0226
	Short inside	do	Leaves	11.64	.987	3.98	.9052	.0103	.0257	.0012	.0312
			Shoots	12.48	.723	5.44	.9517	.0088	.0310	.0043	.0279
Previously rosetted, treated. [3]	Top	do	Leaves	5.88	.403	1.70	.2734	.0068	.0129	.0014	.0074
			Shoots	6.74	.205	2.45	.3368	.0058	.0115	.0019	.0285
	Side	do	Leaves	8.75	.718	2.89	.4997	.0094	.0149	.0009	.0103
			Shoots	7.86	.128	3.28	.5420	.0044	.0095	.0019	.0190
	Short inside	do	Leaves	12.84	.887	4.90	.8163	.0102	.0193	.0007	.0111
			Shoots	13.82	.590	7.11	.7722	.0086	.0293	.0036	.0403

[1] Samples of the topmost leaves of this tree had severe chlorosis, the topmost shoots showed some necrosis, the outside branches had chlorotic leaves but no necrosis, and the inside branches were virtually free of rosette symptoms.

[2] This healthy tree was treated by placing 30 g of commercial zinc sulphate ($ZnSO_4 \cdot 5H_2O$) in its trunk June 22, 1933.

[3] This tree was rosetted prior to June 22 when it was treated as was the healthy tree (see note 2); it was healthy when sampled.

TABLE 2.—*Percentage ash and certain mineral constituents in tissues from the tree shown in fig. 1 which was treated on July 11, 1933*

[Samples for analysis collected Aug. 18, 1933]

Position of branches	Treatment	Condition	Tissue	Ash	Silica	CaO	MgO	Mn	Fe	Cu	Zn
Top	Untreated	Mild rosette	Leaves	6.66	0.527	1.96	0.5350	0.0088	0.0195	0.0010	0.0068
			Shoots	6.52	.237	2.41	.3877	.0042	.0156	.0016	.0202
			Average	6.59	.382	2.18	.4613	.0065	.0175	.0013	.0135
	Treated	Healthy	Leaves	5.21	.425	1.33	.3946	.0058	.0161	.0009	.0849
			Shoots	5.47	.261	1.69	.3048	.0043	.0153	.0009	.0749
			Average	5.34	.343	1.51	.3497	.0050	.0157	.0009	.0799
Side	Untreated	Mild rosette	Leaves	7.05	.476	2.06	.5813	.0081	.0207	.0008	.0074
			Shoots	7.85	.372	2.94	.5587	.0068	.0216	.0016	.0207
			Average	7.45	.424	2.50	.5700	.0075	.0211	.0012	.0140
	Treated and girdled.	Healthy	Leaves	8.51	.789	2.59	.6926	.0119	.0248	.0008	.0258
			Shoots	7.88	.241	3.11	.5127	.0041	.0148	.0010	.0478
			Average	8.20	.515	2.85	.6026	.0080	.0198	.0009	.0368
	Treated and defoliated.	do	Leaves	5.72	.647	1.37	.4895	.0059	.0203	.0011	.0361
			Shoots	6.79	.712	1.92	.4220	.0043	.0028	.0020	.0435
			Average	6.25	.679	1.65	.4557	.0051	.0115	.0015	.0398

ASH AND MINERAL CONTENT OTHER THAN ZINC

The data throw light upon the mineral content of healthy and rosetted pecan trees, especially with regard to the consistent difference in ash content between different types of shoots or parts of the tree

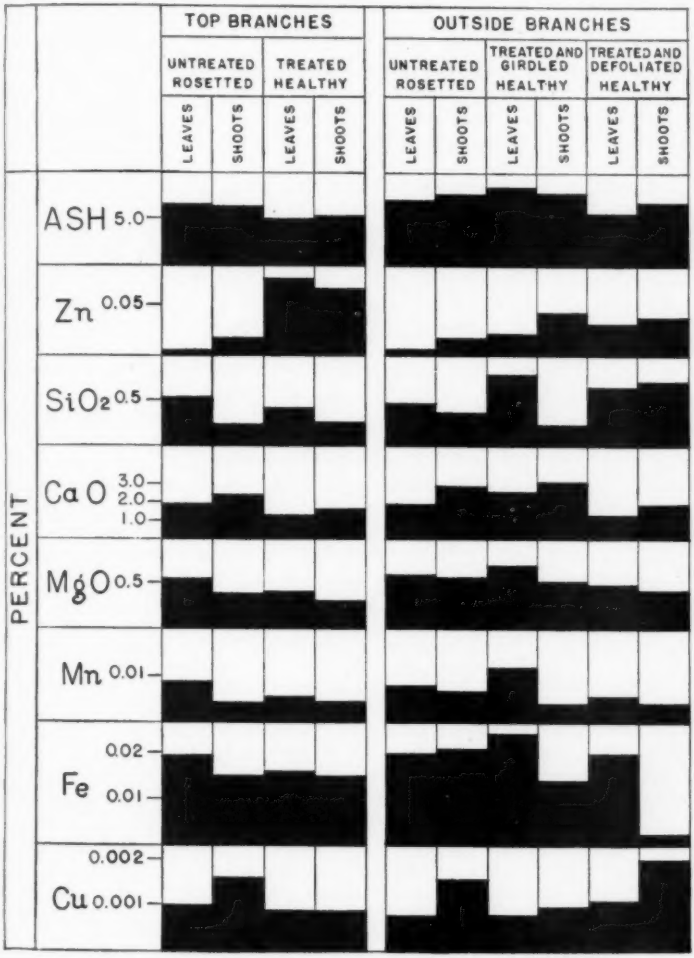

FIGURE 3.—Results of analysis of the tree given special treatment as shown in figure 1.

(table 1 and fig. 2). Short inside branches in the lower part of the tree had consistently a greater ash content than had the long growths in the top of the tree. The outside shoots, which are intermediate in length and vegetativeness, were generally intermediate in percentage of ash.

Along with the difference in mineral content of branches from different parts of the tree, there appears to be a relation between development of rosette and ash content. The top branches, the most severely affected part of the rosetted tree, had a much higher ash content than had comparable samples from healthy trees (table 1 and fig. 2). In the tree given special treatment (table 2 and fig. 3) the treatment of one top branch with zinc (fig. 1) and the subsequent production of healthy growth was accompanied by a reduced ash content. The data of 1934 (table 3 and fig. 4) are also indicative of a higher ash content in rosetted than in comparable healthy tissue. Haas (*12*) has reported a higher ash content in rosetted than in healthy walnut and pecan tissue.

However, in the rosetted tree (table 1 and fig. 2) the short inside shoots which were entirely free of rosette symptoms were higher in ash than were the rosetted top shoots. It seems that the ash content is influenced by the condition of growth and location in the tree. The increased ash content found for rosetted tissue may be the result of rosette and not present in a causal relation. It is only when growths of similar type or vegetativeness are considered that the relation between ash content and rosette symptoms is apparent.

Further evidence that the mineral content is influenced by the amount or quality of the growth as well as the health of the tissue is seen in the results following girdling and defoliation (table 2 and fig. 3). The growth produced after girdling had a somewhat increased ash content; that produced after defoliation had a reduced ash content. Following girdling, the shoots were short and otherwise resembled those on the inside of the tree. Following defoliation, the growth was longer, slender, and willowy, approaching the character of the healthy topmost shoots where the ash content was low. Samples were of growth following defoliation and girdling and both were of the same age.

TABLE 3.—*Percentage of ash and zinc content of leaves at top of rosetted and healthy trees of several varieties in the Yuma Valley*

[Collected Oct. 16, 1934] [1]

Variety	Condition	Ash	Zinc
Kincaid	Rosetted	8.96	0.003
		8.01	.0071
		7.73	[2]
		7.36	[2]
	Healthy	7.37	.0202
		6.11	.0136
		6.24	.0153
Halbert	Rosetted	8.06	.0053
		7.84	[2]
		7.63	[2]
	Healthy	5.07	.0157
		5.54	.0174
		5.34	.0066
		7.71	.0048
Success	Rosetted	8.50	[2]
		7.43	[2]
	Healthy	6.59	.0163
		6.03	.0145
		6.38	.0084

[1] The rosetted trees were on the east side of the valley where rosette is somewhat common. The healthy trees were on the west side of the valley where it is unknown.

[2] Traces.

What was true of ash content has been generally true for each of the elements determined except zinc. A comparison of the analyses of the topmost tissue in the healthy untreated and in the rosetted tree indicates that silica, calcium, magnesium, manganese, and iron may be higher in rosetted than in comparable healthy tissue. At the same time, the rosetted top shoots were for the most part no higher in these materials than were the healthy, short inside shoots. For the rosetted tree alone, the latter were highest in the various elements. There is a general trend in the data from all of the healthy trees for the short, inside shoots to be highest in all elements and for the outside shoots to be intermediate. Similarly, in the untreated, girdled, and defoliated outside shoots, the calcium, magnesium, manganese, and iron were reduced in the defoliated tissue and were high or increased in the girdled or untreated tissue. Copper appears to bear little relation to condition of health or type of growth.

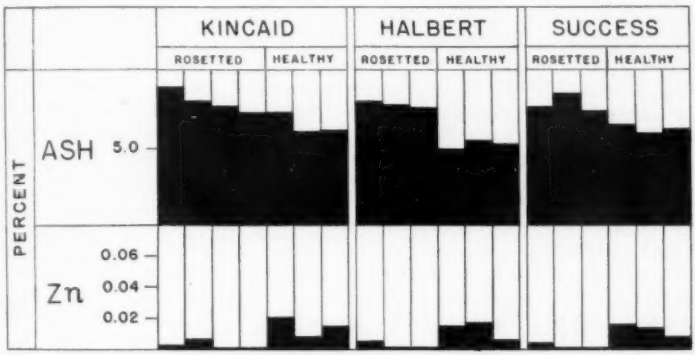

FIGURE 4.—Ash and zinc content in topmost leaves of rosetted and healthy pecan trees in the Yuma Valley, collected October 15, 1934. These trees represented what are believed to be wider differences between a healthy and rosetted condition than those of figure 2.

ZINC CONTENT

The zinc content found in the various tissues exhibits somewhat more of a specific relationship to the presence or absence of rosette symptoms than do the other elements determined. This is particularly true of the samples in table 3 and figure 4. These samples, collected from comparable parts of healthy and rosetted trees, indicated a higher zinc content in the healthy than in the diseased tissue. These healthy samples were from an area in which rosette has never been known, whereas the affected ones were from an area where rosette has been common. Greater differences in fundamental factors responsible for rosette might be expected than in the samples (table 1 and fig. 2) which were all from a locality where rosette has been serious.

In the analysis of 1933 (table 1 and fig. 2) the zinc content found for the untreated rosetted and healthy trees was significantly different only in the short inside shoots where it was markedly less in the rosetted than in the healthy tree. The top branches of these two, which differed widely in degree of health, and the outside branches, which were mildly affected in the rosetted tree and unaffected in the

healthy tree, contained practically the same percentage of zinc. Though the total zinc content was not greatly different, the proportion of zinc to total ash was much reduced in the rosetted tissue.

Data from the rosetted tree alone (table 1) presents an analogous condition. The topmost part which displayed the most extreme symptoms of rosette contained virtually the same percentage of zinc as did the healthy, short, inside shoots and the less severely affected outside shoots.

Following the application of zinc the treated rosetted tree produced healthy growth in the top and outside branches. This was accompanied by but a very slight increase in total zinc content over that of the untreated tree (table 1 and fig. 2). The proportion of zinc in the ash was greatly increased. It would appear that if pecan rosette is related to quantitative amounts of zinc in the tissue, the differential between healthy and affected tissue is small. There is also the possibility that when small amounts of zinc are present, its action is modified by the presence of other minerals occuring in the ash. Chandler, Hoagland, and Hibbard (*5*) have reported some overlapping of zinc content between healthy and little-leaf tissue, generally more zinc being found in the healthy tissue.

In the healthy, untreated tree (table 1) where zinc might be expected to have a normal distribution within the tree, the short inside shoots contained markedly more zinc than the top or side branches. These shoots were from the lower part of the tree. Such shoots are not commonly affected with rosette, even in trees where the topmost parts are badly diseased. While the movement of zinc following treatment is most rapid to the top and rapidly growing parts, its normal accumulation seems to be greatest in the lower, slowly growing branches. Perhaps this is why they are less susceptible to rosette. However, other conditions are not equal, for the upper part of the tree is more exposed to light and heat.

Different parts of trees all equally healthy in appearance have been found to contain widely different amounts of zinc. Apparently after a minimum requirement is satisfied, additional quantities of zinc serve no further need. The appearance of healthy trees was not affected by the addition of zinc.

The absence of a quantitative relation between amount of growth and zinc may indicate that it does not enter extensively into the protoplasmic structure. Reed and Dufrenoy (*19*) suggest that it may act in regulating the oxidation-reduction potential within the cells of citrus leaves. They believe that for an appreciable amount of oxidation, "the formation of complexes with metals which readily change their valences, thereby serving for H and O transfers" is necessary. "Two or more molecules of amino acids may be linked with one of a metal such as iron, copper, manganese, or zinc."

That a metal which readily undergoes a change in valence could be important physiologically as an oxygen acceptor and thereby function in maintaining the oxidation-reduction potential of the cell is a tenable theory. However, zinc is not such a metal. According to Mellor (*16*) zinc belongs to a family of elements (group 11 in periodic arrangement) of which only mercury readily undergoes a change in valence. Even here the lower oxide is unstable. Zinc

forms the monoxide after the formula: $2Zn+O_2=2ZnO$. In this the valence is typically 2. The only other oxide of zinc is the peroxide having the chemical structure:

In the peroxide the valence of zinc is unchanged. In forming this compound, oxygen rather than zinc undergoes a change in valence. Zinc being bivalent could act as a nucleating element joining two molecules of amino acids or similar groups, as Reed and Dufrenoy point out, but judged by the known properties of zinc, there could be no auto-oxidation of the amino acids resulting from valence changes at the union. Such a function for zinc cannot be postulated, as it might well be for iron, copper, or manganese.

Zinc has been reported by several workers to be necessary for the growth of green plants. It has also been known for many years to be beneficial to the growth of fungi. The sum total of the knowledge of zinc in plants is yet small. The present data do not indicate that any large amount of zinc is held in affected tissue unavailable for physiological processes. Rather, they would tend to suggest that rosette symptoms are produced when the total zinc in the tissue is insufficient for normal metabolism. Data previously presented (*8*) support this view as do those of Chandler, Hoagland, and Hibbard (*5*) and Reed and Dufrenoy (*19*) for other horticultural plants. Also, substantiating this interpretation is the fact that in earlier studies (*8*), acids injected into affected trees did not bring about recovery through making available any elements or ions already present. Similarly, the application of sulphuric acid to the soil around affected trees in a district where rosette is serious was not effective. It has previously been reported that no relation between soil alkali or salt content and rosette occurred in Arizona (*8*). The occurrence of pecan rosette on the acid soils of the South has been reported by Skinner and Demaree (*20*) and others. Studies of the hydrogen-ion concentration of healthy and rosetted leaves and stems have not revealed consistent differences in reactions.

It seems evident that if any portion of zinc in the tissue is not available for metabolism, it is a much smaller portion than is the case with some other elements. Iron, for example, may be more abundant in tissue, chlorotic for lack of utilizable iron, than in healthy tissue. The greater solubility of zinc may account for its being more readily available. The ultimate success of controlling pecan rosette through treatment with zinc will depend considerably upon whether only supplying a deficiency is necessary or whether measures to influence its availability in the tree are also involved.

This matter may also have bearing upon the fluctuation and irregularity of occurrence of rosette. If zinc is not held unavailable in appreciable quantities in the tree, then the development of the disease on healthy trees would point to soil fixation. It has been frequently observed in Arizona that where corrals or chicken pens have been built around trees, rosette soon follows. Chandler et al. (*5*) have reported that corral soils have a high zinc-fixing power.

MOVEMENT OF ZINC IN THE TREE

The treatments given the tree shown in figure 1 were designed especially to give information upon the movement of zinc in the tree. This was deemed important especially in view of the erratic occurrence of rosette wherein one limb of a tree may be affected and the rest healthy, and wherein the top of the tree is almost always more severely affected than the lower parts.

The samples collected from the top branches 35 days after one branch had been treated with zinc revealed wide differences in zinc content (table 2 and fig. 3). The average zinc content in the tip of the treated branch was approximately six times that in the untreated. In the latter, the content was not increased above that for comparable tissue in the untreated trees (table 1). Since it is impossible to know the zinc content of the same tissue both at time of treating and subsequent thereto, it cannot be known if a small increase occurred in the untreated branch. There had been some slight improvement in the health of the untreated branch, and it is possible that the zinc content had increased slightly. It is evident, however, that the greatest movement of zinc was upward in the treated branch and that little moved downward to the junction with the untreated branch and thence upward in it.

The zinc content found in the lower branches supports this interpretation. The treatment in one lower branch (table 2 and figs. 1 and 3) did not increase the amount of zinc found in the outside shoots of the untreated branch on the opposite side of the tree, beyond that found for the outside branches of the untreated trees (table 1). Here again, severe rosette symptoms had not developed, nor had they developed anywhere in the tree. This fact is taken as indicating some migration of zinc downward from the point of treatment to the trunk where it joined the general vascular supply of the tree. If such a migration downward occurred, the amount was not great enough to be measured. Clearly, the most immediate and abundant movement of zinc is in an acropetal direction. In the field treatment of many trees, it has been observed that limbs above the treatment are the first to respond, but the entire tree eventually does so.

The severely rosetted condition of one branch of an otherwise healthy tree may be accounted for by the tendency of zinc to move upward in the tree. In such cases, one might suspect the absorption of zinc by the roots below the affected limb.

The healthy tree treated with zinc in 1933 revealed an increased zinc content over the healthy untreated tree only in the top and outside branches. The increase was greater in the side branches. There was no increase in the short inside branches. This might be interpreted as chemical evidence of a fact many times observed in the treatment of rosetted trees with zinc; the topmost shoots, while most severely affected are first to recover, and rapidly growing shoots respond more quickly than slowly growing ones. In young, vigorously growing trees the response of zinc applied to the trunk has often been noted on young leaves in 3 days following treatment. Zinc applied in excess amounts has produced a toxic effect on the leaves in 24 hours.

Girdling of a limb on the low branch (fig. 1) did not prevent the passage of zinc from the point of treatment upward, for the sample

from above the girdle contained markedly more zinc than did the outside branches of any of the trees sampled. The movement of zinc upward must then be in the xylem. The new shoots on the defoliated limb likewise were found to have an abnormally high zinc content. Seemingly, the reduction in leaf area adjacent to the new growths did not affect the movement of zinc into them.

SUMMARY

The present paper reports additional evidence that the occurrence of pecan rosette is to a considerable extent associated with an insufficiency of zinc for normal metabolism in the tissue. Such factors as condition of growth, location in the tree, exposure to light and heat, and probably many others may act with a reduced zinc content to cause the development of rosette symptoms. No evidence was found that any appreciable quantity of zinc is present but not utilizable in affected tissue.

The total ash content was found to bear a relation to the occurrence of rosette when shoots of similar growth character and position in the tree were compared. In comparisons of this kind, the ash of affected tissue was found to be higher than that of healthy tissue. Since other factors influence the ash content so markedly, we cannot look upon it as occurring in a causal relation to pecan rosette. It is more probably a result.

The translocation of zinc occurred in the xylem. Zinc, when placed directly in the xylem, moved most rapidly in an acropetal direction. A slower lateral and downward migration probably occurred.

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THE ISOLATION OF A HEMOPHILIC BACILLUS IN PURE CULTURE AND THE REACTION OF CHICKENS TO EXTRANASAL INOCULATIONS THEREOF [1]

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INTRODUCTION

The study of hemophilic bacilli herein reported deals with two different phases: (1) A technique of isolating *Hemophilus gallinarum* in pure culture; and (2) the reaction of chickens to the extranasal inoculations thereof.

ISOLATION OF HEMOPHILIC BACILLI IN PURE CULTURE

In a previous publication (*2*),[2] the writers reported their success in isolating hemophilic bacilli from the edematous facial swellings of chickens showing an infectious coryza. The technique consisted in employing several loopfuls of the edematous fluid from the swellings to inoculate a blood medium. After 24 hours incubation at 37° C. the microscopic examination of the medium usually showed a pure growth of these organisms.

Since the time of that report, an opportunity of studying infectious material from other than the source used in the previous work has become available, and the technique has been found applicable to that type of infection with which edematous swellings about the face are commonly observed.

Certain difficulties were experienced in isolating the organisms directly from the edematous facial swellings of fowls from field outbreaks, but susceptible chickens, infected under laboratory conditions with material from these field cases and examined within 24 hours after swellings were noted, yielded pure cultures from each of the various field sources of the disease. It is apparent that the duration of the infection in the individual fowls from the field outbreaks was unknown and since, in the previous work of the writers, the cultures had been made when the swellings were first noted, the time factor seemed to be of significant importance in obtaining positive results.

De Blieck (*1*), Nelson (*4*, *5*, *6*), Schalm and Beach (*7*), and Eliot and Lewis (*3*), have described their method of isolating the organisms from the nasal exudate. They have also shown the significance of these organisms in producing a respiratory infection in susceptible fowls. In the writers' experience, the isolation of the organisms from the nasal exudate is somewhat tedious and more difficult to accomplish than is isolation from the swellings, although it probably constitutes the only manner in which they may be isolated in those cases showing only a nasal exudation.

[1] Received for publication Oct. 1, 1935; issued April, 1936. Published as Contribution No. 474 of the Rhode Island Agricultural Experiment Station.

[2] Reference is made by number (italic) to Literature Cited, p. 383.

MATERIAL AND METHODS

Preparation of Media

The blood was obtained directly from the heart of chloroformed fowls after the keel and ribs had been cut in such a manner as to expose the heart and was collected in a sterile glass hypodermic syringe and placed in a sterile 2-percent sodium citrate normal salt solution at the rate of 5 cc of blood to from 10 to 20 cc of this solution. In this manner from 15 to 20 cc or more of sterile whole blood could be obtained from the average fowl.

When media were desired for use, the citrated blood was added to nutrient agar slants so that it extended from three-fourths to 1 inch from the bottom of the slant. It was found desirable to use fresh blood and make up the media just before they were used.

Method of Isolation

The skin over the edematous swellings of the fowls to be examined was seared, and an incision through the seared skin was made with a hot instrument. Two to three loopfuls of the edematous fluid were used to inoculate each of several tubes of the media as described above.

After these had been incubated at 37° C. for 24 hours, smears were made from each culture tube and stained with Gram's stain. The microscopic examination of stained smears constitutes the only sure way of detecting growth as the changes occurring from growth in this medium are only slight except for the coagulation of the blood portion which may, at times, occur within this period.

A modification of Gram's method of staining may be used to good advantage. It consists in substituting gentian violet made as follows: A, 4 g of gentian violet in 25 cc of 95-percent alcohol; B, 14 cc of aniline oil and 2.5 cc of hydrochloric acid in 500 cc of distilled water. The iodine solution is made up of a 1–1,000 solution of tincture of iodine in water. This stain is used in the usual manner except that the safranin is allowed to stain from 1 to 2 minutes or longer. With this stain the smear has a pink background, and the bacteria stain a more intensive pink.

CULTURAL AND MORPHOLOGICAL CHARACTERISTICS OF HEMOPHILUS GALLINARUM

The morphological and cultural characteristics of *Hemophilus gallinarum* have been described by the writers in a previous publication (*2, pp. 7–8*). The organisms colonize on blood-agar slants and plates if grown in an atmosphere containing sufficient moisture (*2*), or in an atmosphere containing 10 percent of carbon dioxide as reported by Schalm and Beach (*7*).

Stained preparations from blood-agar slants or plates usually show the organisms to be larger than those grown in the blood-citrate-salt solution at the base of nutrient agar slants.

Thus far, attempts to grow the organisms on other than a blood medium have not been satisfactory, although growth in a medium consisting of small pieces of sterile raw potato in broth and sodium citrate solutions at the base of nutrient agar slants have shown scanty growth as indicated by stained preparation of the media.

Pure culture of the organisms grown in blood at the base of nutrient agar slants produce swellings when inoculated subscutaneously in chickens and also cause a systemic reaction as indicated by the birds appearing depressed.

Stock cultures maintained in the blood at the base of nutrient agar slants have been found capable of inducing respiratory symptoms when inoculated into chickens after a period of 10 months although the virulence is greatly reduced at this time.

EXPERIMENTAL DATA

Bacteriological examinations were made of the edematous material from facial swellings of 10 field cases from 5 sources. *Hemophilus gallinarum* was isolated from only one bird representing a field case of the infection.

Eight of nine susceptible fowls inoculated from the respective field infections gave positive results when the material from their edematous swellings was used to inoculate the same type of medium within 24 hours after the swellings were first noted. The other fowl was not examined.

As nothing was known with respect to the length of time the infection had persisted in any of the individual field cases, this factor seemed to be of considerable significance in the successful isolation of the organisms. Tests were therefore conducted with a series of adult Barred Plymouth Rock and White Leghorn chickens which were inoculated intranasally to determine the practicability of isolating organisms from fowls within 24 hours after the swellings about the face were noted, and also after 48 hours.

The bacillus was isolated from five of the six birds within the 24-hour period, whereas the results were negative in five of the six fowls examined at the 48-hour period.

The results would indicate that the bacilli of *Hemophilus gallinarum* are fewer in numbers or have ceased to grow at some period 24 hours after the swellings have developed. This probably runs parallel to the course of the infection, for on an average the swellings ordinarily persist for only a few days and recovery is usually apparent within a week or 10 days from the onset of the infection in the particular strains studied.

THE REACTION OF CHICKENS TO THE EXTRANASAL INOCULATIONS OF HEMOPHILIC BACILLI

The ability of certain hemophilic bacilli to induce an inflammation of the upper respiratory tract of chickens has been reported by De Blieck (*1*) Nelson (*4, 5, 6*), Delaplane, Erwin, and Stuart (*2*), Schalm and Beach (*7*), and Eliot and Lewis (*3*). A certain degree of resistance to reinfection has been noted in chickens that have recovered from the field type of infection. These chickens are particularly resistant to the intranasal inoculations of cultured organisms, while those recovering from culturally induced infection are quite resistant to reinfection with the same cultured organisms but are not completely protected against the disease as found in the field.

Extranasal inoculation of cultured organisms was made to note the reaction in chickens and to determine whether such a procedure would induce resistance to intranasal infection with the same strain of organisms.

In a preliminary small group of chickens, it was noted that the subcutaneous inoculation of freshly isolated cultured organisms was followed by swellings 24 hours after inoculation. These birds were quite lame and considerably depressed for a few days but subsequently recovered and were found resistant to intranasal inoculations of the cultured organisms and the field infection 2 weeks after the subcutaneous injections had been made.

MATERIAL AND METHODS

The chickens constituting the various experimental groups were of different breeds and ages at the time of use but each group of birds was of the same breed and age. They were reared on the poultry farm at the experiment station and were known to have been free of clinical evidences of respiratory infections prior to experimentation.

The culture material used for the various groups was 24-hour cultures grown in a blood citrate solution and was diluted just prior to use with sterile normal salt solution.

The subcutaneous inoculations were made in the skin over the leg, a 20-cc syringe and an 18-gage needle being used. The skin area at the point of injection was painted with tincture of iodine before and after the inoculations were made. The technique for the intraperitoneal and wattle inoculations was similar to that used for subcutaneous inoculations in that the point of inoculation was prepared in a similar manner.

EXPERIMENTAL DATA

The primary tests were conducted on a group of adult Rhode Island Red pullets. Six birds received the cultural organisms subcutaneously in the skin area over the leg, and three were injected in the wattles. The two groups were maintained in separate quarters. Twenty-four hours following the inoculations, swellings were evident at the point of inoculation of both groups. The six birds receiving the subcutaneous injections were considerably depressed and lame for a few days but gradually became normal. Two weeks later they were inoculated intranasally with material from a transfer of the same culture used for the injections. They were resistant to this intranasal inoculation. Still later these same birds were inoculated with the nasal exudate from fowls which had been infected with the exudate of the field form of the disease, and from which the organisms had been originally isolated. They were found resistant to this field form of the disease. The control chickens obtained from the same source as the ones used for the tests were susceptible to the cultured organisms and the field infection. The three birds that had been injected in the wattles contracted intranasal infection within 72 hours after the injection and were not tested after they recovered.

The results of the preliminary tests were so encouraging that it was planned to conduct the experiment on a larger scale. Two groups of White Leghorn pullets consisting of 40 birds each comprised the initial groups. The two groups were maintained in separate pens and each received 0.5 cc of a 50 percent 24-hour culture solution subcutaneously over the right leg. One group received the organisms from culture 1,[3] and the other from culture 2.[4] They were quite

[3] Culture isolated from the Rhode Island type of this disease.
[4] Cultures obtained from other than Rhode Island sources.

depressed over a period of 5 days after the inoculations had been made, and a number were lame in the leg receiving the inoculation.

Twenty days following the subcutaneous inoculations a few of the birds of each group which had not been inoculated intranasally or been in contact with cases of the disease, contracted intranasal infection. Following the observation of the first few cases, the remainder of each group developed a coryza.

The effect of the intranasal inoculation of 19 of the fowls which had been inoculated with culture 1 was tried after they had recovered from the subcutaneous inoculations and the subsequent intranasal infection which they had contracted. All the birds except one were found resistant to reinfection from cultures 1 and 2.

The intranasal reinoculations of eight of the birds originally inoculated with culture 2 were made after they had recovered from the subcutaneous inoculations and the subsequent intranasal infection. They were all resistant to inoculations of cultures 1 and 2.

Ten 4-week-old Barred Plymouth Rock chickens were used in a study of the effect of the subcutaneous inoculation. These chicks were confined in a battery brooder in isolated quarters, and special precautions were taken to be sure that any leakage from around the needle at the time of inoculation was promptly sponged with cotton saturated with tincture of iodine. These precautions were taken to avoid accidental intranasal infection from extraneous material.

These birds received two injections of culture 1, the second being made 10 days after the first. Each of the chickens showed swellings at the point of inoculation 24 hours following each injection. They were somewhat depressed and lame for a period of 5 days following the first inoculation but did not appear to have reacted so severely as the adult birds. These chicks contracted intranasal infection 23 to 25 days after the first inoculation in spite of the precautions taken to make sure that any material leaking from around the needle could be at fault, thus indicating that intranasal infection might occur from the subcutaneous inoculation of cultured material.

The reaction of the 10 birds to intranasal reinoculation was also tested after they had recovered from the intranasal infection. Two of the chickens contracted intranasal infection as a result of the reinoculation with cultured organisms of culture 1.

Five Rhode Island Red pullets were injected intraperitoneally with cultured material on October 29 and November 1 and were maintained in adjoining separate cages of a laying battery. These birds did not show any appreciable reaction to the intraperitoneal injections. Four of them contracted intranasal infection 9 days after the first inoculation.

It was considered of interest to determine whether the combining of cultured organisms with laryngotracheitis virus and inoculating the material in the cloaca would offer any protection. Five 6-week-old Barred Plymouth Rock chickens were inoculated in the cloaca with this material, culture 39 being used with the laryngotracheitis vaccine. Four days after the inoculation of the combined material, all chicks showed extensive cloacal reactions. None of the chicks contracted laryngotracheitis or intranasal infection as a result of the inoculation. Nine days later they were inoculated in the larynx with laryngotracheitis vaccine and culture 39 and were found resistant.

Five days later they were inoculated intranasally with cultured organisms, and after 24 hours they all showed intranasal infection, showing that no resistance had developed from the cloacal injections 2 weeks after the inoculation.

SUMMARY AND CONCLUSIONS

Evidence is presented to show that *Hemophilus gallinarum* may be readily isolated in pure culture from the edematous swellings of fowls showing an infectious coryza and that the success of the isolation depends on the length of time the swellings have persisted. Thus, if the material is cultured within 24 hours after the swellings have developed, the isolation of the organisms is readily accomplished. After 48 hours, there is less chance of obtaining the organism. As the duration of time the infection has persisted in field cases is usually unknown, it is suggested that for diagnostic purposes susceptible fowls be inoculated intranasally with the infective material and cultures made from them within 24 hours after the facial swellings are noted.

The technique serves as an aid in the differentiation of certain other respiratory infections of fowls in which a coryza is a symptom, particularly that type characterized by a marked rattled breathing and lesions of an acute bronchitis and tracheitis with which a coryza is noted and which may also show some swelling around the eye. This particular infection is perhaps the same as that described by Beaudette and Hudson [5] at the Northeastern States Pullorum Disease Research Workers Conference. The infective agent of this disease readily passes through Berkfeld V and N filters as indicated by the ability of these filtrates to produce the typical disease condition in susceptible fowls. In contrast the filtrate of infectious coryza exudate does not produce infection when inoculated into susceptible fowls.

The extranasal inoculation of chickens with cultures of hemophilic bacilli is attended with reactions at the points of inoculation when given subcutaneously or in the wattle. The birds also show a systemic reaction as evidenced by those appearing depressed. The intraperitoneal inoculation of a small group of birds did not seem to result in any appreciable difference in their general health.

Intranasal infection occurred as a result of the subcutaneous injection of the cultured organisms. It would be difficult, however, to state the percentage that contracted the infection in this manner, since each of the birds of an experimental group was in contact with the others and when one developed intranasal infection, it was easy for the others to become infected from it.

No particular resistance to intranasal infection was noted in the birds receiving subcutaneous inoculations of cultured organisms, although approximately 3 weeks elapsed from the time of inoculation until they contracted intranasal infection.

The intranasal reinoculation of the birds recovering from the subcutaneous inoculations and the subsequent intranasal infection showed that all but one bird of the Leghorn-pullet groups and two from the Barred Plymouth Rock group to be resistant to reinfection.

[5] Unpublished data.

Injection of the cultured organisms in the wattle was followed by intranasal infection. Four of five birds receiving intraperitoneal injections likewise contracted intranasal infection within 9 days after receiving the first inoculation.

The cloacal inoculation of the cultured organisms combined with laryngotracheitis virus did not result in any upper respiratory infection in a group of five chickens. The chickens were later found to be resistant to laryngotracheitis virus, but susceptible to intranasal inoculations of cultured organisms.

The fact that the preliminary group was resistant to intranasal reinoculations remains unexplained.

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CHROMOSOME BEHAVIOR IN BLACKBERRY-RASPBERRY HYBRIDS [1]

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INTRODUCTION

The genus *Rubus* in the United States is characterized by interspecific compatibility, frequent lack of complete fertility, and a high degree of heterozygosity. This is shown by the taxonomic work of Brainerd and Peitersen (*2*)[3] on the New England blackberries, by the genetic studies on the same material by Peitersen (*10*), and by several investigations of Darrow and Longley (*4*) and of Longley (*5*, *6*). This is further emphasized by the dewberry × raspberry cross made by Ness (*9*) in 1912. Both first and second generations

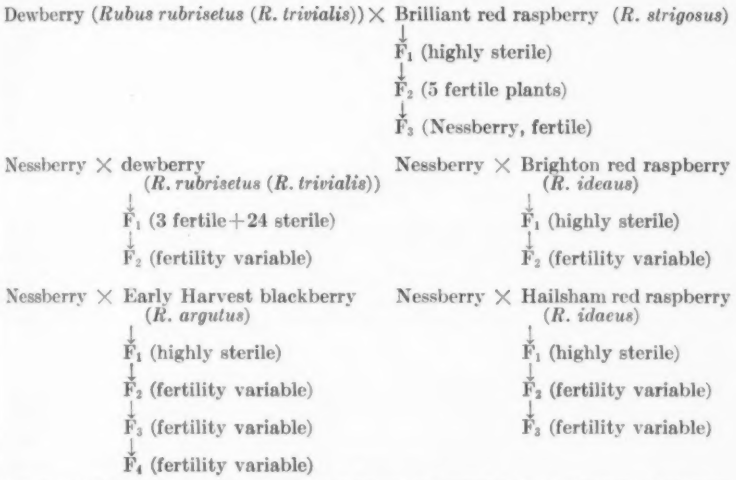
Dewberry (Rubus rubrisetus (R. trivialis)) × Brilliant red raspberry (R. strigosus)
F1 (highly sterile)
F2 (5 fertile plants)
F3 (Nessberry, fertile)
Nessberry × dewberry (R. rubrisetus (R. trivialis))
F1 (3 fertile+24 sterile)
F2 (fertility variable)
Nessberry × Brighton red raspberry (R. ideaus)
F1 (highly sterile)
F2 (fertility variable)
Nessberry × Early Harvest blackberry (R. argutus)
F1 (highly sterile)
F2 (fertility variable)
F3 (fertility variable)
F4 (fertility variable)
Nessberry × Hailsham red raspberry (R. idaeus)
F1 (highly sterile)
F2 (fertility variable)
F3 (fertility variable)

FIGURE 1.—Diagrammatic representation of the origin and later crosses of the Nessberry with blackberry, dewberry, and raspberry.

of this cross (*Rubus trivialis* Michx. = *R. rubrisetus* Rydb. × Brilliant red raspberry, *R. strigosus* Michx.) were quite sterile with the exception of five F_2 (open-pollinated) plants, which were almost completely fertile, giving a third generation of 859 plants. Selections from these were given the name Nessberry. Ness then crossed this fertile hybrid to the dewberry parent, to two raspberries, and a blackberry and grew later generations. The last generation of each cross with the Nessberry, outlined in figure 1, has been available for cytological study.

[1] Received for publication Aug. 26, 1935; issued April, 1936.

[2] The writer acknowledges the assistance of Fraser Cowart, Dr. U. R. Gore, and Hennie Levy in the preparation of cytological material at different times.

[3] Reference is made by number (italic) to Literature Cited, p. 396.

The work here reported was undertaken to obtain further knowledge of chromosome behavior in the genus *Rubus*.

Since all of the crosses were made by the late Helge Ness, with whom the writer had no opportunity to discuss the material, portions of his notebook entries will be given in certain instances. An account of much of this work has been published by Ness (*8*, *9*). In referring to his publications it must be remembered that he named the pollen parent first. For example, his paragraph heading (La × (Brilliant × La) F_3) F_1 thus becomes the first generation of the backcross Nessberry × *Rubus rubrisetus* (*R. trivialis*) (*9*).

THE WILD DEWBERRY

Ness (*9*) considered *Rubus trivialis*, the dewberry found locally, and *R. rubrisetus*, secured for his work from Louisiana, as distinct species, based upon differences in shoot growth. It is doubtful, in view of the amount of variation in this respect observed in dewberries of this locality, if there is sufficient genetic difference between the two to justify this distinction. Root tips of the former have 14 chromosomes at metaphase (fig. 2, *A*). There are seven pairs at diakinesis, frequently joined by a single terminal chiasma. The movement toward the poles at the anaphase of the first division sometimes tends to be a little uneven (fig. 2, *B*), otherwise the division appears normal.

THE NESSBERRY

Longley and Darrow (*7*) illustrate the heterotypic division of the Nessberry with three figures. Diakinesis and metaphase exhibit 14 pairs of chromosomes; 1 pair appears to be lagging at telophase. They say: "It was exceptional to find irregularities during meiosis, but the history of this hybrid shows that it has been selected through several generations and now behaves like a stable species." A slightly greater degree of irregularity than the foregoing account would indicate has been observed in plants grown in Texas. Lagging has been noted during the heterotypic division, and in addition the chromosomes seem to have some difficulty in forming a normal metaphase plate. This interferes with an even movement toward the poles during the anaphase of the first division (fig. 2, *C*).

NESSBERRY × RUBUS RUBRISETUS F_2

The first generation of the cross Nessberry × *Rubus rubrisetus* is discussed by Ness (*9*, *pp. 22–23*). The cross was described before it was known that the Nessberry has twice the chromosome number of its parents. This first generation consisted of 27 plants—

> intermediate of the parents, except three individuals which show only the Nessberry in all parts and bear a heavy crop of perfect fruits; all the others are more or less sterile, with canes showing traces of both parents. * * * The leaves have the form and size of the Nessberry but their texture and surface are characteristic of the blackberries. The flowering is abundant, but each resulting fruit consists of a few scattered drupelets.

The simplest explanation of these results would seem to be that 3 tetraploid plants of the maternal type and 24 triploid true hybrids had been obtained. None of this generation has been available for study.

A second generation was grown on two occasions. Seed was sown on October 15, 1924, from F_1 plants of the Nessberry type. Five of

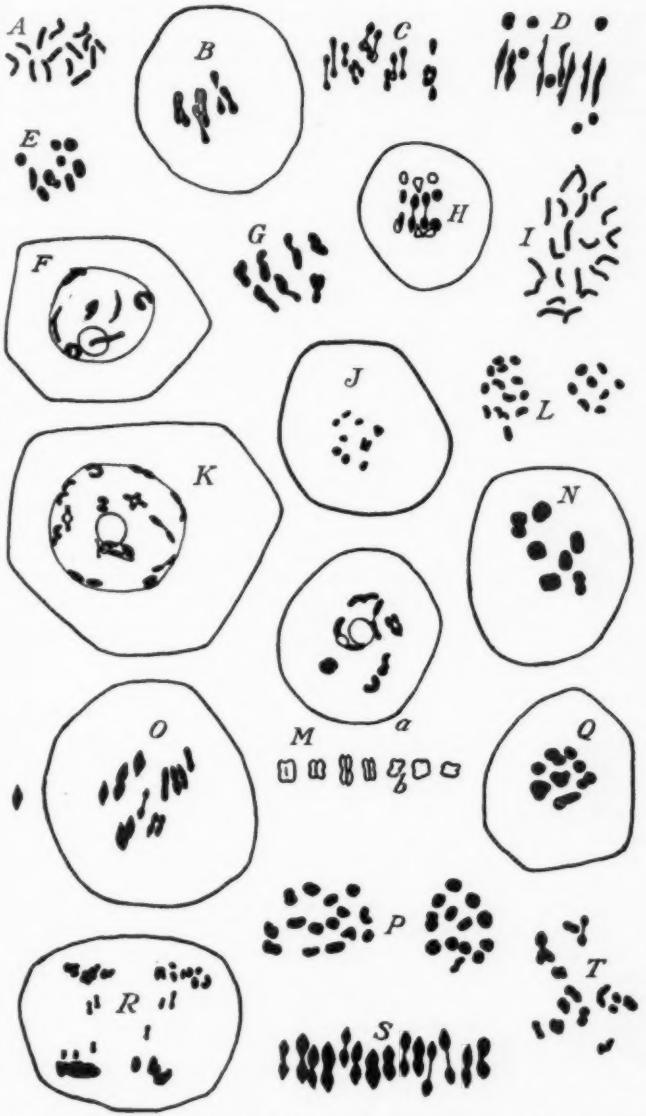

FIGURE 2.—*A*, *Rubus trivialis*, root tip. *B*, *R. trivialis*, first anaphase; *C*, Nessberry, first anaphase. *D*–*J*, Nessberry × *R. trivialis* F_2: *D*, 3*n*, first anaphase; *E*, 3*n*, first metaphase; *F*, 2*n*, diakinesis; *G*, 3*n*, first metaphase; *H*, 2*n*, first anaphase; *I*, 3*n*, root tip; *J*, 3*n*, first metaphase. *K*–*R*, Nessberry × Hailsham (*R. idaeus*) F_2, 4*n*; *K*, diakinesis; *L*, second metaphase; *M*, *a*, diakinesis; *M*, *b*, first metaphase; *N*, first metaphase; *O*, first anaphase; *P*, second metaphase; *Q*, first metaphase; *R*, second anaphase. *S*–*T*, Nessberry × Early Harvest (*R. argutus*) F_4, 4*n*; *S*, first anaphase; *T*, first metaphase-anaphase. × 2,540. Buds and root tips were fixed in Allen's modification of Bouin's solution and paraffin sections made, which were stained in crystal violet. In the buds only pollen mother cells were examined. All drawings were made with the aid of the camera lucida, using a 1.5-mm apochromatic oil-immersion objective and a 12.5 × compensating ocular.

the seventy-one plants are mentioned in the notes. In four of these the Nessberry parent was considered to be dominant; the fifth had "strong growth, many flowers but infertile, similar in all parts to the F_1", presumably the intermediate type. The second planting was made October 12, 1927, "Seeds uniformly large." The following February it was noted that the germination was poor, 64 plants being potted. Sixty-eight plants were set in the field during May 1928. Forty-two of these have been available for observation.

The plants may be classified roughly into four groups: (1) A single plant in which the characters of the Nessberry parent are dominant; (2) a variable intermediate group of 10 plants; (3) those almost indistinguishable in appearance of canes from the dewberry parent, 28 plants; and (4) 3 plants similar to those just preceding except that they are more vigorous and show Nessberry characters in the canes. The plants will be discussed in this order.

(1) No. 27 resembles the Nessberry parent very strikingly in type of growth, i. e., the leaflets are large and broad, usually in threes; the canes are strongly branched, with fewer prickles and less pigmentation than the wild dewberry. In addition, the ripe fruit, which is reduced in size because of sterility, crumbles upon picking instead of breaking below the receptacle as in *Rubus trivialis*. Twenty-one chromosomes were observed in root-tip preparations (fig. 2, *I*). There is evidently normal pairing between 14 chromosomes while 7 remain unpaired. This can be seen in the heterotypic anaphase illustrated in figure 2, *D*. Assortment of the unpaired chromosomes is probably at random. Counts of a rather limited number of metaphase plates of the homotypic division indicate that 9 and 12 is the most common distribution, followed by 8 and 13 and then 10 and 11. In figure 2, *L*, the distribution has been 8 to one pole and 13 to the other; one of the chromosomes has split and the halves are about to disjoin.

The 10 plants of the intermediate group (2) differ widely in vigor. Six are highly sterile, the other four having reduced fertility. No. 1, sterile but vigorous, has the tetraploid chromosome number, with complete pairing. Three or four tetrasomes can be observed at diakinesis. Two chromosomes frequently lag at anaphase of the first division. No. 8, sterile and weak, has the diploid chromosome number, which is readily seen at diakinesis (fig. 2, *F*). While the seven pairs seem to behave quite normally, evidence of a loose association between the two components of one of the disomes was noted. No. 12 is fairly fertile; the 14 pairs frequently exhibit a multiple association with from 1 to 6, rarely 7, tetrasomes at diakinesis. The distribution is usually 14 and 14, but 13 and 15 chromosomes are sometimes observed at the homotypic metaphase. Judging from premetaphase and first anaphase stages, plant no. 29 has the tetraploid chromosome number. Chromosomes of certain of the hybrid plants are hard to differentiate clearly, and since no. 29 is one of these, the precise chromosome relationships are hard to determine. There are at least 12 pairs in this case, with a variable amount of multiple association during diakinesis and first metaphase, resulting in 7 or 8 groups of chromosomes in some instances. No. 29 appears to be less fertile than no. 27. The other six plants of this group were not examined cytologically. A summary of chromosome counts for this and other crosses is given in table 1.

An examination has been made of the pollen mother cells from six plants which bear a close resemblance to the dewberry parent (group 3). A count was made of the chromosomes in the root tips of an

additional plant. In each case the diploid number was found. These plants give evidence of their hybrid origin by their vigor, by the immense size of the fruits in many cases, by a peculiar flavor of the fruit of certain of the plants, and in some instances by a reduced fertility. The pairing is complete, although somewhat looser than in *trivialis*. There is a reluctance of one or two chromosome pairs to disjoin promptly (fig. 2, *H*); otherwise the divisions are quite regular. There was a large amount of apparently good pollen—68 and 78 percent for two plants examined in iodine-potassium iodide solution.

TABLE 1.—*Summary of chromosome counts obtained*

Cross	Group no.	Type	Plants				
			2*n*	3*n*	4*n*	Not examined	Total
			Number	*Number*	*Number*	*Number*	*Number*
Nessberry × *R. rubrisetus* (dewberry) F_1	1	♀ P dominant		1			1
	2	Intermediate	1		3	6	10
	3	♂ P dominant	7			21	28
	4	♂ P predominates, vigorous		3			3
		Total	8	4	3	27	42
Nessberry × Brighton (raspberry) F_2	1	Dewberry type				2	2
	2	Nessberry type			2	4	6
		Total			2	6	8
Nessberry × Hailsham (raspberry) F_2	1	Entirely fertile			27	46	73
	2	Fertility reduced			28	29	57
	3	Sterile			7	16	23
		Total			62	91	153
Nessberry × Early Harvest (blackberry) F_1	1	Entirely fertile			18	62	80
	2	Reduced fertility			29	38	67
	3	Sterile			5	27	32
		Total			52	127	179

The last group (4) consists of three plants differing from those immediately preceding by their greater vigor, reduced pigmentation and number of prickles, and increased branching of the canes. All three have the triploid chromosome number. Classification of chromosome groups of an early first metaphase of no. 45b on a basis of size suggests three monosomes, three disomes, and four trisomes (fig. 2, *E*). A spindle view of first anaphase, not illustrated, lends support to this interpretation.

The chromosome number of no. 45c was determined from root-tip material only. Both somatic and meiotic chromosomes of no. 55 were examined. There is a strong tendency for the formation of trisomes in the latter plant, as can be seen during the early first metaphase of figure 2, *G*. There are not usually so many trisomes. In figure 2, *J*, also a first metaphase, there is only one obvious trisome, at the upper right.

NESSBERRY × BRIGHTON F_2

Nessberry × Brighton represents a backcross to a raspberry species, *R. idaeus*, L., similar to the original parent. The first generation was discussed by Ness (*9*, *p. 23*). It was noted that—

> All of the plants set a profusion of flowers, but not a single flower produced perfect fruit. Each fruit consisted, as in other cases of partial sterility, of only

a few drupelets. By gathering nearly every drupelet, an ample amount of seed for a second generation was secured (*9, p. 25*).

There were 230 plants, all no doubt with the triploid chromosome number.

Seeds for the second generation were sown in boxes on October 12, 1927; "germination very poor." Eleven plants were potted the following March, and 12 plants were set in the field on May 15, 1928. Eight of these have been available for study. In spite of the use in the breeding work of large numbers of insect-proof cages large enough to cover a plant, it seems highly probable that this family resulted from open pollination, as two of the plants are quite similar to the wild dewberry except in fertility. Three plants of the Nessberry type are almost completely sterile and three plants are quite fertile. Two of the latter have the tetraploid chromosome number. Fourteen pairs can be seen at diakinesis and in both cases there is evidence of a multiple association to give anywhere from one to six loose tetrasomes. Lagging occurs at both heterotypic and homotypic anaphases. One of these had 34 percent apparently good pollen.

NESSBERRY × HAILSHAM F_2

Nessberry × Hailsham is the same type of cross as the one just discussed, the two first-generation families being very similar. Seed for the second generation was saved from a plant "with perfect, large fruit, foliage differing from rest in texture * * * very similar to Nessberry in all characters." The F_2 consisting of 93 plants, was planted in the field in 1925. Notes were taken the following May on 10 plants. Two were of the Hailsham type, but completely sterile; one was like the wild dewberry, but with "imperfect" fruits; the remainder varied, both in appearance and fertility. On May 16, 1927, an additional plant was described as follows: "Low, compact; leaves, shoots typical of Nessberry; very prolific; flower cluster large; berries large, Nessberry in form and color; drupelets adhering; tendency to break under calyx." While no record is available, this unusual praise, together with the appearance of this third generation, makes it seem quite likely that this plant was its parent. Two hundred and seventy F_3 plants were set in the field on May 14 and 15, 1928. One hundred and fifty-three of these have been available for observation.

With the exception of certain plants of weak growth the family in general favors the Nessberry. All plants have been placed in three groups on a fertility basis, as follows: (1) Entirely fertile, 73 plants; (2) fertility reduced, 57; (3) almost or entirely sterile, 23. The classification is based upon the appearance of the fruit. Buds of 60 plants have been examined. As far as could be determined from pollen mother cell material all plants have the tetraploid chromosome number. The somatic chromosome number of one of these was found to be 28.

While the first group seemed to be completely fertile as judged by achene development, in nine representative plants the percentage of good pollen varied from 45 to 67. In addition, no. 72 had only 16 percent of good pollen, while, in contrast to this, no. 7 had only 3 percent of empty grains, the remainder varying greatly in size, none staining as deeply as pollen from other plants.

Three of the twenty-seven plants examined exhibited no multiple association. Most of the plants had one or two tetrasomes at diakinesis. In four cases as many as seven tetrasomes were sometimes found (fig. 2, *N*), and in one plant six tetrasomes and two disomes. Two of the plants having seven tetrasomes were at first mistaken for diploids (Yarnell (*11*)), but further examination showed that they as well as the others have the tetraploid number of chromosomes. In spite of an evident reluctance to form a normal plate at first metaphase with consequent lagging of chromosomes during the anaphase following, the usual division is 14 chromosomes to each pole. The great variation in size of pollen grains found in no. 7 is probably due to the loss of lagging chromosomes. The low percentage of good pollen in plant no. 72 was not fully explained by a study of meiosis. There was some evidence of a tendency toward incomplete association at diakinesis but this was rare.

The chromosome behavior of the plants with reduced achene development differs from that of the foregoing group only in degree. The percentage of good pollen from six representative plants was found to vary from 36 to 50, somewhat below that of the previous group. As before, two exceptional plants were found—no. 47 with 85 percent of good pollen and no. 162 with no good pollen.

The diakinesis shown in figure 2, *K*, has 14 pairs of chromosomes. This is from a plant (no. 74) having from none to seven tetrasomes. The figure illustrated in figure 2, *O*, represents a first anaphase of this same plant, and illustrates disjunction without the formation of a metaphase plate. One pair covered by others is drawn outside the cell. The diakinesis of figure 2, *M*, *a*, has seven tetrasomes, which are shown in figure 2, *M*, *b*, as they appear at first metaphase. A polar view of the first metaphase of this plant (fig. 2, *Q*) has five tetrasomes and four disomes. Lagging at second anaphase (fig. 2, *R*) occurs in the group.

The last group was originally classified as: (1) Almost sterile, 13 plants; (2) entirely sterile, 3 plants; and (3) weak growth with no flowers, 7 plants. Three plants were found to have the following percentages of good pollen: 3, 21, and 24, respectively. The amount of multiple association varies from none to seven tetrasomes as before. The lack of organization at the normal period for first metaphase is more pronounced, however. This results in nondisjunction more frequently than in the more fertile plants. A 15 and 13 division (fig. 2, *P*) is fairly common.

NESSBERRY × EARLY HARVEST F_1

Nessberry × Early Harvest represents an outcross to a blackberry type. Early Harvest is said to have been propagated from a selected wild plant growing in southern Illinois. It is referred to *Rubus argutus* Link by Card (*3*) and by Bailey (*1*). The cross was made in 1919, the small first generation having black fruit rather than the deep red of the Nessberry. In leaves and stem the plants favored the female parent. The flowers were highly sterile. Seeds to produce the second generation were "medium to small, of Early Harvest type." On May 3, 1923, 10 plants were set in the field. On May 19, 1924, there were "seven plants, intermediate except no. 6 and very uniform in characters." One of the intermediate plants was chosen

as parent of the third generation. There was an abundance of both large and small seeds. Sixty-one F_3 plants were set in the field during November 1925. Notes were made on seven of these plants during May 1927. All were fertile and prolific, but they varied somewhat in character of vegetative growth. Seed was saved from two of these for a fourth generation. The seeds were variable in size; germination was good, 336 plants being set in the field in May 1928.

The plants as a whole do not differ greatly in appearance from those of the previous cross, as the Nessberry influence is dominant in both. In each case there is a great deal of variation, particularly in vigor, in size of leaves and flower clusters, and in general habit of growth, including the amount of branching and the degree of procumbency.

A classification of the fertility of the 179 plants available for study was made from the fruit as before. (1) Eighty plants seemed to be entirely fertile; (2) 67 had reduced fertility, and (3) 32 were almost sterile. Pollen was examined from 11 plants of the "fertile" group. They fell into three classes: Six plants with from 70 to 80 percent of good pollen, four plants with from 57 to 59 percent of good pollen, and one plant with 32 percent of good pollen. Ten of thirteen plants examined critically gave evidence of multivalent association, usually having two or three tetrasomes, although two plants had as many as seven tetrasomes part of the time. The other three plants had only disomes at diakinesis and metaphase of the first division. The drawn-out anaphase resulted in an equal distribution of chromosomes in all cases noted. In figure 2, *T*, the chromosomes are separating without the formation of a metaphase plate. There is a single tetrasome at the upper left. This illustrates an extreme degree of irregularity for this group.

Eight of nine plants with "reduced fertility" had from 64 to 82 percent of apparently good pollen. One other plant, no. 23, had only 1.7 percent of good pollen. Buds were fixed from 23 plants of this second group. No. 23 gave evidence of the association of four chromosomes to give two disomes at the metaphase of the second division. From two to four tetrasomes were usually observed during the first division. Anaphase of the first division of this plant was more regular than for most plants of the group (fig. 2, *S*). The chromosomes have been spread in drawing. Considering all 23 plants examined, there were more tetravalent bodies than in the previous group. Only one plant exhibited disomes. The number of tetrasomes commonly found ranged from two to four, with four plants sometimes having as many as six or seven. There was more lagging near the end of the first division in this than in the preceding group. A division of 13 and 15 was observed in six plants; five spores were sometimes formed from one sporocyte.

The six plants classified as "almost sterile" that were examined cytologically differed but little from the foregoing group. The irregularities were of the same type and appeared to be of about the same degree. One plant was found to have 70 percent of apparently good pollen.

DISCUSSION

As pointed out by Ness (*9*) and later by Longley and Darrow (*7*), the five fertile plants of the second generation of the cross wild dewberry (*Rubus trivialis*) × Brilliant red raspberry (*R. strigosus*) behave

as a true species. Since the fertility is due to chromosome doubling in the hybrid F_1, the Nessberry should be classed as an amphidiploid rather than as a true tetraploid. A second cross made by Ness with the same dewberry by Haymaker, *R. neglectus* Peck (*occidentalis* × *strigosus*), appears to have doubled its chromosome complement in a first-generation plant in the same way. Plants of this fertile second generation were fertile with the Nessberry (*9*).

It is of interest to note that there were only five fertile plants among a second generation otherwise highly sterile. Since the first hybrid generation must have had two genoms of seven chromosomes each it appears that the doubling most likely occurred at a time to produce both egg and pollen grains of an F_1 plant with two genomes—probably in a somatic cell during differentiation of a flower bud, possibly giving a sectorial chimera. If it had happened earlier a larger number of fertile second-generation plants would be expected; if later, sterile $3n$ plants would result. Some, indeed, may have had the triploid chromosome number. It is possible, of course, that these five plants were produced from the union of nonreduced gametes. This explanation seems to be less likely than the first because seeds from first-generation plants were sown 2 years in succession without the appearance of a single fertile plant.

The four families under consideration represent backcrosses to the two parental species and an outcross to a distinct species, *Rubus argutus* (fig. 1). The first-generation plants of these later crosses would normally be expected to have the triploid chromosome number. This expectation seems to have been realized in each case with the exception of three plants of the backcross to the dewberry, which were fertile and of the maternal type—possibly due to accidental self-pollination.

In two cases plants of a second generation of backcrosses to the two parental species have been available for study. Twenty-eight of the forty-two plants of the cross involving *Rubus rubrisetus* (*R. trivialis*) have a marked resemblance to this species. All of these plants examined are diploid. The large number of plants of this type suggests that they may have originated by the open pollination of F_2 plants with pollen from the wild dewberry, the egg contributing a haploid chromosome set. All exhibit hybrid characteristics and so must contain chromosomes, or at least portions of chromosomes, derived from the original red raspberry parent. In spite of the wide genetic differences between corresponding chromosomes (being derived from different sections of the genus) they are able to pair in these plants and behave otherwise in a fairly normal manner. The raspberry genes are obviously in the minority since the appearance of the plants is in striking contrast to that of the F_1 of the cross *R. trivialis* × Brilliant, which also must have been diploid. Ness (*9*) reports that the raspberry type was very dominant in the F_1.

The triploids in the backcross under consideration are of two types, one plant being very similar to the Nessberry, and three plants favoring the dewberry but considerably more vigorous. The appearance of these types can be accounted for by supposing that they carry two genoms most or all of whose genes come from the parent they favor. If the latter three plants result from pollen from *Rubus trivialis*, the egg would then have contributed two sets of chromo-

somes, a part of which must have been derived from *R. strigosus*. If association can be taken to represent genetic similarity in this case one would assume that there are two complete genoms and possibly four additional chromosomes that should be referred to *R. trivialis* and three chromosomes deriving largely from *R. strigosus*. In the case of the plant resembling the Nessberry parent one is tempted to assign two genoms to *R. strigosus* and one to *R. trivialis*.

Since the Nessberry regularly has 14 pairs, it is reasonable, considering the wide genetic differences between the parental species, to suppose that pairing is between genoms from the same species, with a resulting lack of opportunity for their modification by crossing over between chromosomes of the two species involved. The first generation of the backcross to the dewberry parent would then have two genoms derived from *Rubus trivialis* and one from *R. strigosus*. Plants with the triploid chromosome number obtained upon selfing a triploid of this constitution would be expected to favor the wild dewberry. The origin of no. 27, favoring the Nessberry is hard to explain. Its appearance, together with the *Drosera* type of pairing observed, strongly suggests that its chromosomal complement does consist of two genoms from *strigosus* and one from *trivialis*.

Plants with the tetraploid chromosome number appear in both second-generation families. This is also probably true in the F_2 of the crosses of Nessberry with the Hailsham raspberry and Early Harvest blackberry, since a portion of plants of these second generations were highly fertile. Selection of plants of the Nessberry type accounts for the appearance, the fertility, and the chromosome number of the later generations studied.

The meiotic chromosome behavior of these later generation 28-chromosome plants differs from that of the Nessberry in two particulars. The association of four chromosomes is characteristic of the heterotypic division and the degree of irregularity is much greater in the former group of plants. The first may result from the accumulation of more than two homologous chromosomes upon the formation of $4n$ plants from $3n$ individuals. The second is the natural result of the introduction of chromosomes from a distinct species together with the changed balance just noted.

These conditions account for the variability found in the third and fourth generations under study in regard to general plant vigor and morphological characters. Since these depend upon the particular genetic combination obtaining in each plant and since the chromosomal irregularity is probably dependent upon a more general effect of the chromosome complement, a close correlation between the degree of vigor and chromosome regularity is not to be expected. This seems, in some instances at least, to be true also of fertility and chromosome behavior.

All of the crosses were made by Ness with the object of securing a berry combining the high quality of the red raspberries with the ability of the wild dewberry to withstand conditions of heat and drought, and, of course, the work was done without a knowledge of chromosome behavior in the hybrids. The possibility of chromosome doubling in a hybrid that is highly sterile because of wide genetic differences between the parents is recognized as a means of extending the activities of the plant breeder. The ultimate effect is to complicate the breed-

ing behavior of the material through the use of parents with different chromosome numbers, through the apparent loss of important recessive characters where pairing is only between chromosomes of corresponding genoms in the new amphidiploid, and through the fact that a larger number of chromosomes prevents simple segregation and recombination of characters observed in the original parents. The third and fourth generations of the Nessberry outcrosses discussed above are particularly good examples of the last point. Even in the fourth generation few plants exhibit a really stable chromosomal balance. Practically all characters show some influence of both parents. Where such chromosome doubling has occurred, the breeder may expect a high degree of variability both as to degree of fertility and general somatic characters in using the material in outcrosses to secure more desirable combinations.

SUMMARY

A cytological study has been made of crosses involving the amphidiploid hybrid of a dewberry × red raspberry cross (the Nessberry) with the diploid dewberry parent and with two diploid raspberries and a blackberry.

Plants with the diploid, triploid, and tetrapolid chromosome number were found in the second generation of the dewberry backcross. A single diploid was of intermediate type; 28 diploids strongly favored the dewberry type and may represent outcrosses of the F_1 to the wild species. The triploids were of two types; one plant favoring the Nessberry and three plants nearer to the wild dewberry but more vigorous. Seven disomes plus seven single chromosomes were found during the first division of the first triploid; from three to four trisomes (occasionally more) can be distinguished in the second type.

Plants of the third generation of the Nessberry × Hailsham red raspberry cross and plants of the F_4 of the Nessberry × Early Harvest blackberry cross all had the tetraploid chromosome number. This is probably due largely to selection of plants favoring the Nessberry in the second and third generations. Meiosis of these plants is distinguished from that of the Nessberry by the frequent occurrence of multivalent association to give from one to as many as seven tetrasomes, and by considerably greater chromosomal irregularity during the reduction division.

It seems likely that such association reflects genetic similarity among the associating chromosomes, since there has been an opportunity for more than two corresponding chromosomes of a genom to become segregated in a single plant.

A wide variation in vigor observed among the plants of these later generations is probably due to genetic differences possible in the recombination of chromosomes from the three species involved.

There was some correlation between chromosome regularity at meiosis and degree of fertility, but the most sterile plants did not exhibit a greater degree of chromosome irregularity than many less sterile.

It is pointed out that a doubling of the chromosomal complement in a sterile hybrid greatly complicates its breeding behavior in subsequent outcrosses.

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